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13. ABSTRACT (Maximum 200 Words)
Selective dopamine agonists acting at the D_2 receptor (D_2R) inhibit the toxicity caused by oxidative stress through a novel growth factor dependent and G protein independent signaling pathway. In response to oxidative stress, PC12 cells activate signaling pathways of both homeostasis, as represented by activation of extracellular regulated kinase (ERK) and pro-apoptotic responses as indicated by p53 activation. Individual cells segregate into two populations within the first hour of stress, either showing the activation of ERK or pre-apoptotic p53 activation. The D_2 agonist, bromocriptine caused protein kinase B (Akt) activation in PC12-D_2R cells and the inhibition of either phosphoinositide 3-kinase (PI3-K), epidermal growth factor receptor (EGFR) or c-Src eliminated the Akt activation and the cytoprotective effects of bromocriptine against oxidative stress. Co-immunoprecipitation and EGFR repression by inhibitor or by RNA interference, studies showed a cross-talk between these receptors in mediating the activation of Akt. D_2R stimulation by bromocriptine induced c-Src and EGFR tyrosine phosphoryation. Furthermore, Src tyrosine kinase inhibitor or dominant negative Src interfered with Akt translocation and phosphorylation. We conclude that the predominant signaling cascade mediating cytoprotection by the D_2 receptor involves c-Src/EGFR transactivation by D_2R, activating PI3-K and Akt.

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Dopamine, receptor, Parkinson's, neurotoxin, apoptosis, PC12, Treatment

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Molecular Mechanisms of Dopamine Receptor Mediated Neuroprotection
Annual Report (July 15, 2002 – July 15, 2003) for Award Number DAMD17-99-1-9558

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A. Introduction

Parkinson's disease (PD) is a progressive, neurodegenerative disorder that is characterized by severe motor symptoms, including uncontrollable tremor, postural imbalance, slowness of movement and rigidity. The main pathological hallmark of this disorder is a pronounced loss of dopamine-producing neurons in the substantia nigra pars compacta, which results in a drastic depletion of dopamine (DA) in the striatum, to which these neurons project. In laboratory studies, DA agonists are reported to have neuroprotective actions and recent clinical trials raise the possibility that they may have neuroprotective effects in PD as well. However, the mechanisms underlying agonist-mediated neuroprotection are not clear. We find that the dopamine D_2 receptor can transactivate phosphoinositide 3-kinase (PI3-K) signal transduction pathway and that this signaling can mediate an increased survival of PC12 cells that have been exposed to oxidative stress or trophic withdrawal (Appendix 1). We have determined the oxidative stress activated signaling pathways leading to the apoptosis in PC12 cells (Appendix 2). We have also elucidated the D_2 receptor mediated signaling mechanisms leading to the activation of PI 3-K (Appendix 3). Our findings are clinically and militarily relevant to therapies that might prevent cell loss in PD.

B. Body

Original statement of work and progress

Mechanisms of damage and protection against neural cell death (completed). Results are summarized in Appendix 1 and 2.

Revised statement of work and progress

1: *Determine the pathway by which PI3-K is activated by the D_2 receptor in the presence of bromocriptine.*

We have elucidated the predominant signaling cascade mediating cytoprotection by the D_2 receptor that involves c-Src/EGFR transactivation by D2R, activating PI3-K and Akt. The results are summarized in Appendix 3.

2: Determine whether the D_2 receptor is tyrosine phosphorylated by bromocriptine in PC12 cells.

      Monoclonal antibodies to D_2 receptor was used to carry out the proposed experiments and the results are summarized in appendix 3.

3: Determine the molecular mechanism underlying the agonist-specific activation of PI3-K by the D_2 receptor

      To determine the domain(s) involved in PI3-K activation by D_2R, we introduced mutations in wild type D_{2L} receptor at potential functional domains present in the receptor (Table 1). Mutations were carried out using QuickChange Site-Directed Mutagenesis kit (Stratagene) and confirmed by sequencing. For each point mutation 1-3 substitutions were performed.

Table 1. <u>List of D_2 receptor mutants</u>

No	Position	Original AA*	Substituted AA
1	141	Leu	Ala
2	,,	Leu	Ser
3	142	Tyr	Ala
4	145	Tyr	Phe
5	,,	Tyr	His
6	,,	Tyr	Ser
7	150	Pro	Ala
8	,,	Pro	Leu
9	213	Tyr	Ala
10	295	Tyr	Ala
11	325	Pro	Ala
12	358	Ser	Ala
13	,,	Ser	Cys
14	370	Lys	Ala
15	,,	Lys	Ser
16	371	Ala	Ser
17	436	Tyr	Phe
18	,,	Tyr	Trp
19	438	Leu	Ala
20	,,	Leu	Ser

* Amino acid

      Currently the mutants are being characterized for their capacity to mediate Gi coupling in GTPγS assays when transfected into PC12 cells and for their capacity to mediate PH-Akt-GFP chimera translocation when co-transfected into PC12 cells. We expect all the proposed objectives will be completed by next year.

C. Key Research Accomplishments

- Oxidative stress induces apoptosis in PC12 cells in a concentration and time dependent manner.

- In response to oxidative stress, PC12 cells activate signaling pathways of both homeostasis, as represented by activation of exrtracellular regulated kinase (ERK) and pro-apoptotic responses as indicated by p53 activation.
- Individual cells segregate into two populations within the first hour of stress, either showing the gene induction mediated by activation of ERK or pre-apoptotic p53 activation.
- Changing the level of oxidative stress alters the relative proportion of pro-apoptotic cells at this early time point.
- Dopamine agonists mediate neuroprotection via activation of D_2 receptor against oxidative stress induced cell death in PC12 cells and immortalized nigral dopamine cells.
- D_2 receptor activates PI3-K to mediate the neuroprotective effect of DA agonists.
- DA agonists differ in the relative efficacy to activate the classical G protein pathway and the neuroprotective PI-3K pathway.
- We found large variations in the capacity of different D_2 agonists in mediating neuroprotection.
- The correlation of the efficiacy of a particular agonist for treating the motor symptoms in PD and its neuroprotective activity in the in vitro assay we have developed is poor. Some symptomatically effective D_2 agonists appear to have low neuroprotective potential.
- Bromocriptine caused protein kinase B (Akt) activation in PC12-D_2R cells and the inhibition of either PI 3-K, epidermal growth factor receptor (EGFR) or c-Src eliminated the Akt activation and the cytoprotective effects of bromocriptine against oxidative stress.
- Large number of D_2 receptor mutants were constructed and being characterized.
- Co-immunoprecipitation studies showed that activation of the D_2 receptor induced its association with the EGFR, suggesting a cross-talk between these receptors in mediating the activation of Akt.
- EGFR repression by inhibitor or by RNA interference, eliminated the activation of Akt by bromocriptine. D_2 receptor stimulation by bromocriptine induced c-Src tyrosine 418 phosphorylation and EGFR phosphoryation specifically at tyrosine 845, a known substrate of Src kinase.
- Src tyrosine kinase inhibitor or dominant negative Src interfered with Akt translocation and phosphorylation.
- The predominant signaling cascade mediating cytoprotection by the D_2 receptor involves c-Src/EGFR transactivation by D_2 receptor, activating PI 3-kinase and Akt.
- We also found that the agonist pramipexole failed to stimulate activation of Akt in PC12-D_2R cells, providing an explanation for our previous observations that, despite efficiently activating G-protein signaling, this agonist had little cytoprotective activity in this experimental system.
- Our results support the hypothesis that specific dopamine agonists stabilize distinct conformations of the D_2 receptor that differ in their coupling to G proteins and to a cytoprotective EGFR-mediated PI-3 kinase/Akt pathway.

D. Reportable outcome

Manuscripts:

1. 1.Nair, V. D., Olanow, C. W. & Sealfon, S. C. Activation of phosphoinositide 3-kinase by D_2 receptor prevents apoptosis in dopaminergic cell lines. *Biochem. J.* **373**, 25-32 (2003). (Appendix 1)

2. Nair, V. D., Yuen, T., Olanow, C. W. & Sealfon, S. C. Early single cell bifurcation of pro- and anti-apoptotic states during oxidative stress. (Appendix 2)

3. Nair, V. D. & Sealfon, S. C. Agonist specific transactivation of phosphoinositide 3-kinase signaling pathway mediated by the dopamine D_2 receptor. *Revised Manuscript submitted to J. Biol. Chem.* (Appendix 3)

Abstracts:
1. Nair, V. D. & Sealfon, S. C. (2002) Differential coupling of dopamine D_2 receptor to phosphoinositide 3-kinase mediates anti-apoptosis in PC12 cells. Society for Neuroscience.

2. Yuen, T., Nair, V. D. & Sealfon, S. C (2002). Coordinated apoptosis early gene program induced by oxidative stress in PC12 cells. Society for Neuroscience.

Development of cell lines:
Stable PC12 cell line expressing human dopamine D_2 receptor (PC12-D_2R).

Funding applied for based on work supported by this award:
Studies on D_2 agonists mediated neuroprotection in dopaminergic neurons.
PI – Venugopalan D. Nair
Funding Agency – Bachmann Strauss Dystonia and Parkinson's Foundation.

Employment supported by this award:
Venugopalan D. Nair - Instructor
Karen Said - Research Coordinator

D. Conclusions

Progress during the proceeding year has been excellent. We are likely to acheive two remaining objectives of the proposed research objectives in final year of this proposal. The results promise to establish the foundation for the identification and implementation of DAergic neuroprotective therapy in idiopathic and toxin-induced PD.

APPENDIX COVER SHEET

Appendices 1-3

Activation of phosphoinositide 3-kinase by D_2 receptor prevents apoptosis in dopaminergic cell lines

Venugopalan D. NAIR, C. Warren OLANOW and Stuart C. SEALFON[1]

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Whereas dopamine agonists are known to provide symptomatic benefits for Parkinson's disease, recent clinical trials suggest that they might also be neuroprotective. Laboratory studies demonstrate that dopamine agonists can provide neuroprotective effects in a number of model systems, but the role of receptor-mediated signalling in these effects is controversial. We find that dopamine agonists have robust, concentration-dependent anti-apoptotic activity in PC12 cells that stably express human D_{2L} receptors from cell death due to H_2O_2 or trophic withdrawal and that the protective effects are abolished in the presence of D_2-receptor antagonists. D_2 agonists are also neuroprotective in the nigral dopamine cell line SN4741, which express endogenous D_2 receptors, whereas no anti-apoptotic activity is observed in native PC12 cells, which do not express detectable D_2 receptors. Notably,

the agonists studied differ in their relative efficacy to mediate anti-apoptotic effects and in their capacity to stimulate [35S]guanosine 5'-[γ-thio]triphosphate ([35S]GTP[S]) binding, an indicator of G-protein activation. Studies with inhibitors of phosphoinositide 3-kinase (PI 3-kinase), extracellular-signal-regulated kinase or p38 mitogen-activated protein kinase indicate that the PI 3-kinase pathway is required for D_2 receptor-mediated cell survival. These studies indicate that certain dopamine agonists can complex with D_2 receptors to preferentially transactivate neuroprotective signalling pathways and to mediate increased cell survival.

Key words: dopamine agonist, G-protein, neuroprotection, Parkinson's disease, signal transduction.

INTRODUCTION

Parkinson's disease (PD) is characterized by preferential degeneration of dopamine (DA) neurons in the substantia nigra pars compacta. Inhibition of oxidative phosphorylation, excitotoxicity and generation of reactive oxygen species are considered important mediators of neuronal death in PD [1]. Recent studies suggest that apoptosis may play a role in the loss of DA neurons in PD [2]. The major executioners of apoptosis, caspases, are activated in dopaminergic substantia nigra neurons from PD patients [3,4]. One experimental model for PD uses the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine ('MPTP') to lesion DA neurons. Chronic administration of MPTP has been found to activate caspases [5,6] and to induce apoptosis in the substantia nigra pars compacta of mice [7].

Laboratory studies demonstrate that DA agonists can protect DA neurons in a variety of tissue culture and *in vivo* models of PD [8]. In the clinic, DA agonists have long been employed as an adjunct to levodopa therapy in advanced PD patients who experience motor complication [9]. Prospective double-blind clinical trials have also demonstrated that DA agonists can provide symptomatic benefits for early PD patients with a reduced risk of motor complications compared with levodopa [10,11]. Recent clinical trials have reported that, in comparison with levodopa, DA agonists delay the rate of decline in neuroimaging surrogate markers of nigrostriatal function [12]. These clinical trials raise the possibility that DA agonists may slow the rate of disease progression and are neuroprotective in PD. There is, however, uncertainty as to the mechanisms responsible for these effects and how they might be protective in PD. Proposed mechanisms include levodopa sparing, direct anti-oxidant effects,

stimulation of auto-receptors and inhibition of subthalamic nucleus-mediated excitotoxicity [8]. In addition, some *in vitro* and *in vivo* studies have noted that the protective effects of DA agonists were eliminated when they were co-administered with D_2-receptor antagonists, suggesting that D_2-receptor activation may contribute to the neuroprotective effects observed in these models [13,14].

In order to clarify the contribution of the D_2 receptor to DA-agonist-mediated neuroprotection and to investigate the underlying mechanisms, we studied the effects of DA agonists in a PC12 cell line model system in the presence and absence of DA D_2 receptors. In these experiments, PC12 cells were induced to undergo apoptosis by either oxidative stress or trophic-factor withdrawal. We found that certain DA agonists, but not all, could induce a robust increase in cell survival via activation of the D_2 receptors. Furthermore our results implicate phosphoinositide 3-kinase (PI 3-kinase) in receptor-mediated cell survival and suggest a dissociation between neuroprotective signalling pathways and the G-protein activation classically associated with D_2-receptor signalling.

MATERIALS AND METHODS

Chemicals

[3H]Spiperone (specific radioactivity 99.0 Ci/mmol) and [35S]guanosine 5'-[γ-thio]triphosphate ([35S]GTP[S]; specific radioactivity 1250 Ci/mmol) was from NEN (Boston, MA, U.S.A.). Bromocriptine, pergolide, quinpirole, $R(+)$-7-hydroxy-2-(N,N-di-n-propylamino)tetraline (7-OH-DPAT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). LipofectAMINE™ and Dulbecco's modified Eagle's medium (DMEM) were obtained from Life Technologies (Gaithersburgh, MD, U.S.A.). Pramipexole was a gift from Pharmacia (Kalamazo, MI, U.S.A.). Protein kinase inhibitors PD98059, SB203580, LY294002 and wortmannin were from Calbiochem (La Jolla, CA, U.S.A.). Antibodies specific to phospho-extracellular-signal-regulated kinase (ERK)1/2, ERK1/2, phospho-p38 kinase and p38 kinase were from Cell Signalling Technology (Beverly, MA, U.S.A.). The anti-active caspase-3 antibody was from Promega (Madison, WI, U.S.A.) and anti-rabbit Cy3-conjugated antibody was from Jackson ImmunoResearch (West Grove, PA, U.S.A.).

Cell culture

PC12 cells [15] were maintained in DMEM supplemented with 10 % horse serum/5 % fetal bovine serum in a humidified atmosphere containing 5 % CO_2 at 37 °C. For differentiation, PC12 cells were plated on to collagen-coated plates in DMEM containing 10 % horse serum and 5 % fetal bovine serum and allowed to attach overnight. The cells were then induced to differentiate by growing in DMEM supplemented with 1 % fetal bovine serum and 100 ng/ml nerve growth factor (NGF) for 10–14 days. Nigral DA cell line SN4741 (a generous gift from Dr J. H. Son, Columbia University, New York, NY, U.S.A.) was cultured as described in [16].

Cell-viability assay

The MTT reduction assay is one of the most widely used assays for determining cell viability [17]; it detects living cells, but not dead ones, and the signal generated is dependent on the degree of activation of the cells [18]. PC12 or PC12-D_2R (PC12 cells stably transfected with the human DA D_2 receptor) cells were plated on 96-microwell cell-culture plates (4×10^3 cells/well in 100 μl of medium) and grown for 24 h. Thereafter, H_2O_2 was added either with or without other compounds at the indicated concentrations, and cells were incubated for another 24 h. When the immortalized dopaminergic cell line SN4741 was used the number of cells per well in a microwell plate was 1×10^4 and incubated with 50 μM H_2O_2 at 37 °C for 18 h. MTT solution (10 μl; 5 mg/ml in PBS) was added to the wells, containing 100 μl of medium, and the plates were incubated for 4 h. Thereafter, 100 μl of a solubilization solution (0.1 M HCl in absolute isopropanol) was added and incubated overnight to dissolve the water-insoluble formazan salt. Quantification was then carried out with an ELISA reader at 570 nm using a 655 nm filter as a reference. Data are expressed as a percentage of the untreated controls, and values represent the means $\pm$ S.E.M. from eight microwells from each of four independent experiments ($n = 32$).

Apoptosis analysis

Apoptosis was measured by nuclear DNA staining, caspase-3 immunocytochemistry and caspase-3 activity. Cells were exposed to 200 μM H_2O_2 and fixed by incubating in 4 % formaldehyde for 30 min. The cells were then permeabilized in PBS/0.2 % Triton X-100 for 10 min. The cells were incubated with blocking buffer (PBS/0.1 % Tween 20/5 % BSA) for 2 h at room temperature. Anti-active caspase-3 antibody (diluted 1 : 250) was added and incubated overnight at 4 °C. After washing, cells were incubated with donkey anti-rabbit Cy3-conjugate antibody (diluted 1 : 500)

for 2 h at room temperature. The cells were washed twice in PBS and the nuclei were stained with 1 μg/ml of the fluorescent DNA dye DAPI (in PBS) for 10 min and then washed with PBS. The liquid was drained and the slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.) mounting medium. For caspase-3 activity, 0.5×10^6 cells/100-mm-tissue-culture plates were grown for 24 h and treated with H_2O_2 as indicated. Active caspase-3 was measured using the Caspase-3/CPP32 fluorimetric assay kit (Biovision, Palo Alto, CA, U.S.A.). Enzymic activity was determined spectrofluorimetrically (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA, U.S.A.) by measuring the kinetics of fluorescence increase at excitation/emission wavelengths of 400/505 nm.

Plasmid transfection

To develop the PC12-D_2R cell line, the D_{2L} cDNA [19] was subcloned into pRC/RSV, transfected using lipofectAMINE into the PC12 cells and G418-resistant clones were isolated and screened for [^{3}H]spiperone ligand binding.

[^{3}H]Spiperone and [^{35}S]GTP[S] binding

[^{3}H]Spiperone and [^{35}S]GTP[S] binding assays were carried out essentially as described in [20]. Membranes were incubated with [^{35}S]GTP[S] (0.5 nM), GDP (5 μM) and increasing concentrations of DA agonists at 37 °C for 20 min. Data were analysed by non-linear regression analysis using the Inplot curve fitting program (GraphPad v3.0).

Immunoblotting

PC12-D_2R cells (3×10^6 cells/100-mm plate) were grown for 24 h and following respective treatments, the cells were washed twice with ice-cold PBS and lysed in buffer 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 % Igepal C630, 1 mM PMSF, 1 mM sodium orthovandate, 5 μg/ml aprotinin and a cocktail of protease inhibitors (Roche Diagnostics GmbH) at 4 °C for 20 min. After centrifugation at 14 000 g for 20 min at 4 °C, equal amounts of proteins were resolved by SDS/PAGE. The resolved proteins were electrotransferred to nitrocellulose membrane and incubated with phosphorylated ERK1/2 or p38 kinase antibodies, and then detected with peroxidase-conjugated secondary antibodies and chemiluminescent ECL reagent. The blots were then stripped in stripping buffer containing 62.5 mM Tris/HCl, pH 6.7, 2 % SDS and 100 mM β-mercaptoethanol and probed for total ERK or p38 kinase protein.

Statistical analysis

Data were analysed by either two-tailed Student's t test or ANOVA followed by Tukey's test for multiple comparisons.

RESULTS

Oxidative-stress-induced apoptosis in PC12 cells

We used oxidative stress or withdrawal of trophic support to induce apoptosis in PC12 cells as a model system for studying the putative neuroprotective property of D_2-receptor agonists. Cells were incubated for 24 h with varying concentrations of H_2O_2, and cell viability was assayed using the MTT metabolism assay.
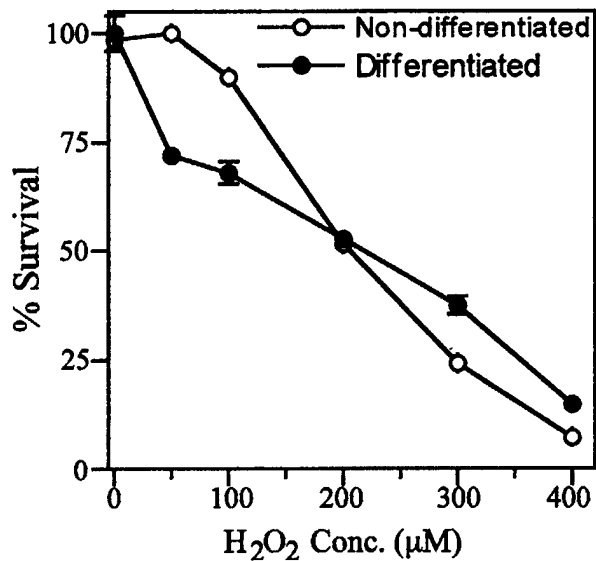
Figure 1   Concentration-dependent cell death induced by H$_2$O$_2$ in undifferentiated and differentiated PC12 cells

Cells were exposed to various concentrations of H$_2$O$_2$ for 24 h and cell viability was assessed using the MTT assay ($n = 8$).

Both undifferentiated and post-mitotic PC12 cells that had been differentiated into a neuronal phenotype by NGF exposure were studied. H$_2$O$_2$ reduced cell survival in a concentration-dependent manner (Figure 1). NGF-differentiated cells were more sensitive than undifferentiated cells to low H$_2$O$_2$ concentrations and were similar in sensitivity at higher H$_2$O$_2$ concentrations. In comparison with untreated controls, cell survival observed in differentiated cells exposed to 50 μM H$_2$O$_2$ was 72.1 ± 4.8 % compared with 99.9 ± 2.4 % in non-differentiated cells ($P < 0.001$). Cell survival observed with 200 μM H$_2$O$_2$ was 52.8 ± 2.2 and 51.5 ± 2.1 % in differentiated and undifferentiated cells respectively.

Apoptotic degeneration is associated with activation of caspase-3-like proteases in *in vitro* models of PD [6,21,22]. To test whether the oxidative-stress-initiated cell death was associated with apoptosis, we evaluated the effects of H$_2$O$_2$ on caspase-3 activation (Figure 2). Results obtained with enzymic caspase-3 assay show that 100 μM H$_2$O$_2$ activates caspase-3 as early as 2 h and that activity continued to increase until 16 h (Figure 2C). With 200 μM H$_2$O$_2$ treatment, maximal caspase-3 activation was observed at 6 h (Figure 2C). These patterns of active caspase-3 were consistent with the greater degree of cell loss observed with increasing concentrations of H$_2$O$_2$ (Figure 1). To confirm that caspase-3 activation in these cells was associated with morphological features of apoptosis, differentiated cells were labelled for caspase-3 activation and stained with the DNA dye DAPI. H$_2$O$_2$ at 200 μM induced the characteristic pattern of chromatin condensation in cells that also stained positive for caspase-3 activation (Figure 2A). Apoptotic changes were evident 8 h after the addition of H$_2$O$_2$, and were noted in 42.2 ± 6.9 % of nuclei after 16 h (Figure 2B).

Differentiated PC12 cells undergo apoptosis in the absence of NGF and serum [23]. After 24 h of trophic withdrawal, cell viability of differentiated PC12 cells was reduced by 48.5 ± 3.0 % compared with control cells ($P < 0.01$). The trophic-withdrawn cells also stained positive for active caspase-3 and showed nuclear fragmentation (results not shown). Collectively, these data indicate that H$_2$O$_2$ or trophic withdrawal causes cell death in PC12 cells by inducing apoptosis.

A



Control   H$_2$O$_2$ (16 h)

B

C
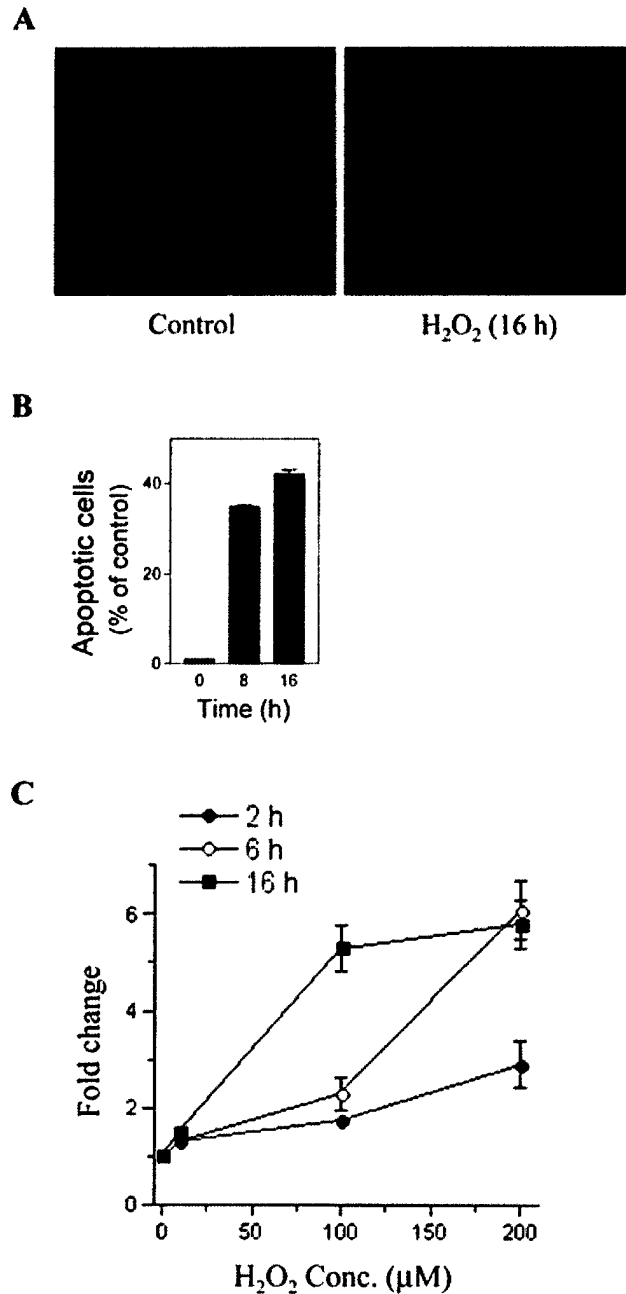
Figure 2   Oxidative-stress-induced apoptosis in PC12 cells (A) and caspase-3 activation induced by H$_2$O$_2$ in differentiated PC12 cells was associated with nuclear condensation

(A) The nuclei were stained blue (DAPI) and active caspase-3, red. Left-hand panel, control; right-hand panel, PC12 cells 16 h following 200 μM H$_2$O$_2$ exposure. Note the association of caspase-3 activation with chromatin condensation. (B) Percentage of apoptotic cells with condensed nuclei and active caspase-3 determined by counting random fields ($n = 6$) at 8 and 16 h following H$_2$O$_2$ exposure, which showed a significant difference ($P < 0.01$) compared with the control. (C) H$_2$O$_2$-induced caspase-3 enzymic activity in undifferentiated PC12 cells was dependent on H$_2$O$_2$ concentration and incubation period ($n = 4$).

D$_2$-receptor activation is required for the prevention of apoptosis in PC12 cells

We studied the potential of DA agonists to protect against apoptosis induced by oxidative stress or by serum and NGF withdrawal in PC12 cells that lacked D$_2$ receptors. The absence of D$_2$ receptors in these cells was demonstrated by the lack of specific
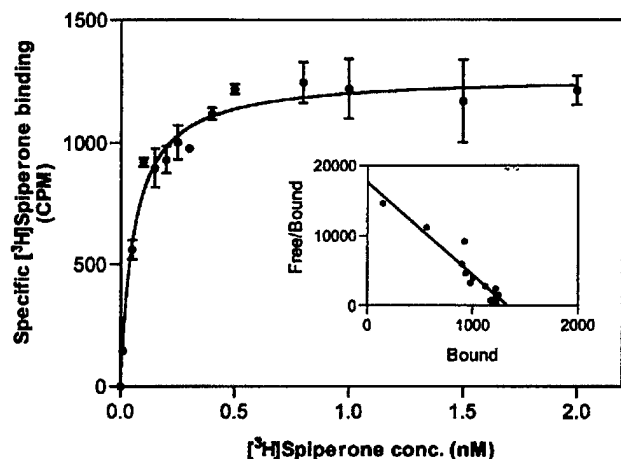
**Figure 3 Saturation analysis for [³H]spiperone binding in membranes of PC12-D₂R cells**

D₂L DA receptors were expressed in PC12 cells and saturation binding analysis using [³H]spiperone was performed to determine levels of D₂ receptors. Binding parameters (K_d and B_{max}) were derived from the data, and values are expressed as the mean ± S.D. values from one experiment performed in triplicate, representative of three independent experiments.

binding using the D₂-receptor ligand [³H]spiperone as well as by the inability to detect D₂-receptor mRNA by PCR (results not shown). The DA agonists tested, bromocriptine and pergolide, provided no protection against apoptosis in either differentiated or undifferentiated cells exposed to H₂O₂ or removed from NGF and serum. These results indicate that these DA agonists were not neuroprotective in the absence of functional D₂ receptors in this model system.

To study the possibility that the DA D₂ receptor might mediate neuroprotection, PC12 cells were stably transfected with the human DA D₂ receptor (PC12-D₂R). PC12-D₂R membranes showed specific [³H]spiperone binding with a B_{max} of 545 ± 14 fmol/mg of protein, a K_d of 0.54 ± 0.07 nM (Figure 3) and competitive binding K_i values for various D₂-receptor agonists and antagonists consistent with reported values for the human D₂ receptor [19]. In PC12-D₂R cells, oxidative stress or withdrawal of trophic support induced cell loss and the stigmata of apoptosis similar to what was observed in PC12 cells that lacked D₂ receptors.
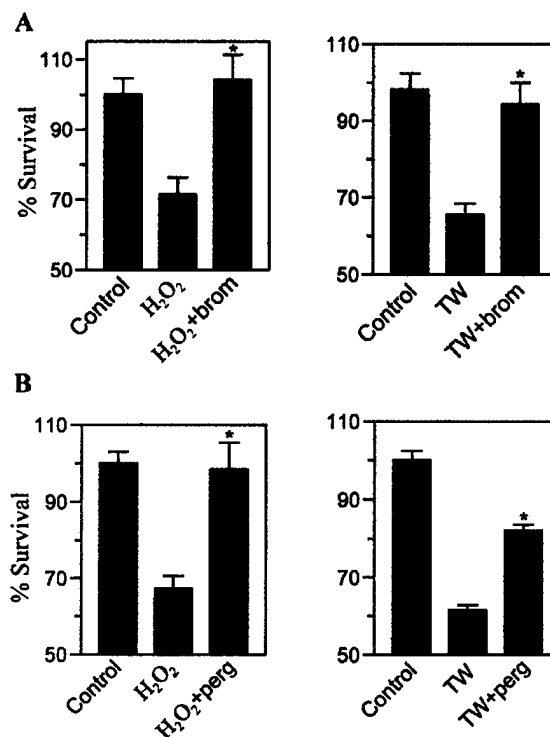
Undifferentiated PC12-D₂R cells were exposed to 200 μM H₂O₂ for 24 h in the presence or absence of various concentrations of the DA agonist bromocriptine, and cell viability was measured. In contrast to the lack of agonist-mediated neuroprotection observed in the parent cell line, bromocriptine dramatically protected undifferentiated PC12-D₂R cells from H₂O₂-induced apoptosis in a robust and concentration-dependent manner (Figure 4A). The protective activity of DA agonists was inhibited by the D₂-receptor antagonists haloperidol and butaclamol, further confirming that the neuroprotection was mediated by activation of the D₂ receptor (Figure 4B). In NGF-differentiated cells, 100 nM bromocriptine and pergolide similarly inhibited apoptosis induced by H₂O₂ and trophic-factor withdrawal (Figure 5).

To explore whether the D₂-receptor activation protects dopaminergic neurons, we investigated the neuroprotection by bromocriptine in the mouse immortalized nigral DA cell line SN4741, which expresses tyrosine hydroxylase, the DA transporter and the D₂ auto-receptors [16]. It has been reported that treatment with 1-methyl-4-phenylpyridinium (MPP⁺), neurotoxins and H₂O₂ equally induces oxidative-stress-dependent apoptotic cell death in SN4741 cells [16,24]. The DA agonist
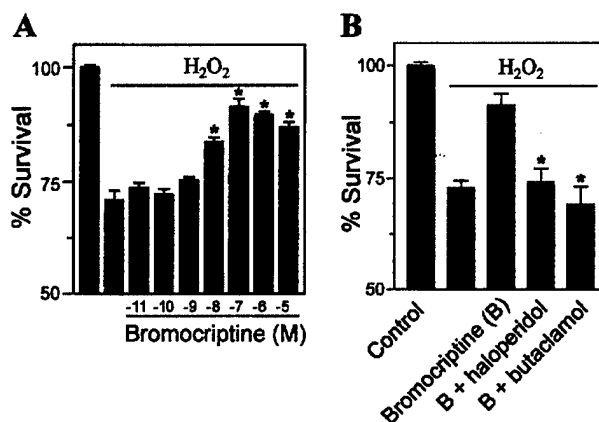
**Figure 4 D₂-receptor activation protects PC12 cells from oxidative-stress-induced apoptosis**

(A) Bromocriptine increased cell survival of undifferentiated PC12-D₂R cells exposed to H₂O₂. Cells were exposed to 200 μM H₂O₂ for 24 h in the presence of increasing concentrations of bromocriptine and cell viability assessed by MTT assay ($n = 8$). *$P < 0.01$ compared with H₂O₂ alone. (B) DA agonist neuroprotection depends on interaction with the D₂ receptor. The protective effect of bromocriptine against H₂O₂-induced apoptosis was eliminated in the presence of the D₂-receptor antagonists haloperidol or butaclamol (1 μM; $n = 8$). *$P < 0.01$ compared with bromocriptine + H₂O₂.

Figure 5 DA agonists protect against apoptosis in differentiated PC12-D₂R cells

Differentiated PC12-D₂R cells were exposed to 200 μM H₂O₂ or trophic withdrawal (TW) in the presence or absence of 100 nM bromocriptine (brom) and pergolide (perg; $n = 8$). *$P < 0.05$ compared with H₂O₂ or trophic withdrawal.

bromocriptine showed significant concentration-dependent increased cell survival against H₂O₂ (50 μM)-induced cell death in SN4741 cells (Figure 6). These results suggest that activation of an endogenous D₂ receptor can be neuroprotective.
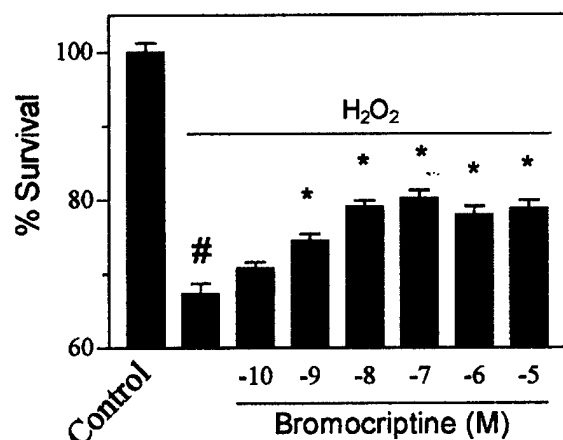
**Figure 6   Bromocriptine protects nigral DA cell line SN4741 from oxidative-stress-induced cell death**

Cells were exposed to 50 μM H$_2$O$_2$ for 18 h in the presence of increasing concentrations of bromocriptine and cell viability was assessed by MTT. Data are mean $\pm$ S.E.M. ($n = 16$). #$P < 0.001$ compared with control. *$P < 0.001$ compared with H$_2$O$_2$ alone.

Differential neuroprotective activity of DA agonists does not correlate with GTP[S] binding

DA agonists varied considerably in their ability to stimulate neuro-protection (Figure 7A). Bromocriptine provided the greatest neuroprotection in this assay, 7-OH-DPAT had low activity and pramipexole was nearly devoid of neuroprotective activity (Figure 7A). The major signalling pathway associated with activation of the D$_2$ receptor involves the heterotrimeric G-proteins, principally G$_i$. The capacity of agonists to stimulate G-protein activation was determined by [^{35}S]GTP[S] binding in membranes from PC12-D$_2$R cells (Figure 7B). Whereas bromocriptine and pramipexole showed marked differences in neuroprotective activity, they demonstrated similar agonist-stimulated [^{35}S]GTP[S] binding and they both exceeded the [^{35}S]GTP[S]-binding activity of pergolide and 7-OH-DPAT. These results indicate that DA agonists differ in their capacity to induce neuroprotection and further suggest that there is a weak correlation between activation of neuroprotective and G-protein signalling pathways.

Neuroprotection by D$_2$-receptor agonists involves the PI 3-kinase signalling cascade

To characterize the molecular mechanisms responsible for the D$_2$-receptor-mediated neuroprotection, we tested the effectiveness of several protein kinase inhibitors against bromocriptine-mediated cell survival in oxidative-stress-induced apoptosis. Inhibition of ERK by PD98059 (50 μM) or p38 kinase by SB203580 (20 μM) had no effect on the increased cell survival caused by D$_2$R activation (Figure 8 and results not shown). In contrast, the D$_2$ receptor-stimulated increase in cell survival required PI 3-kinase activation. The PI 3-kinase inhibitors wortmannin (100 nM) and LY294002 (10 μM) completely abolished the capacity of bromocriptine to protect against oxidative-stress-induced cell death (Figure 8). Control toxicity studies indicated that, at the concentrations used, the inhibitors had no effect on cell survival, as determined by MTT assays (results not shown). However, the inhibitors of ERK and p38 kinase completely inhibited the activation of ERK by epidermal growth factor and p38 kinase by NaCl, respectively (Figure 9). These data suggest that activation of the PI 3-kinase pathway through the
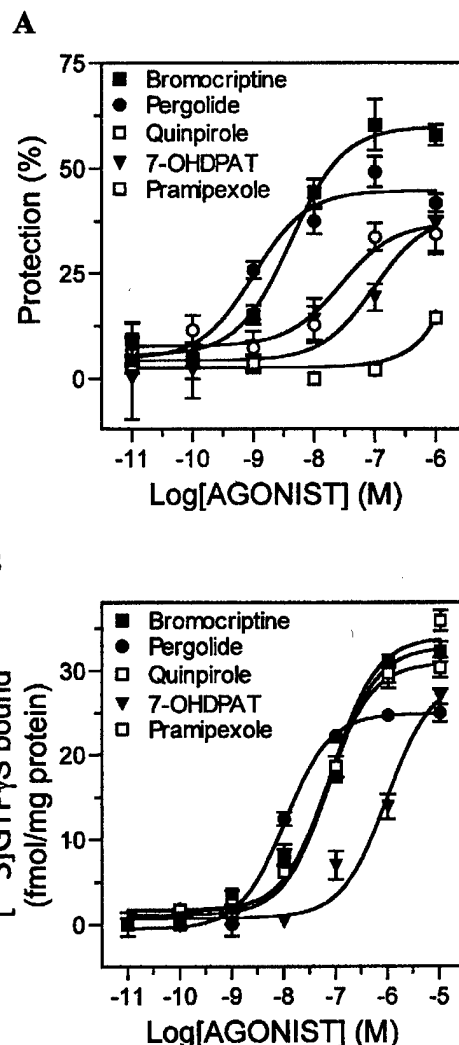

Figure 7   Differential neuroprotective efficacy of DA agonists

(**A**) Comparison of DA agonists in protection of PC12-D$_2$R cells against oxidative stress. Cells were exposed to 200 μM H$_2$O$_2$ for 24 h in the presence or absence of various concentrations of five DA agonists and the effects on cell survival determined by MTT assay. The rank order of protection observed was consistent in three independent experiments. (**B**) Concentration–response curve of the stimulation of [^{35}S]GTP[S]-specific binding to PC12-D$_2$R membrane by DA agonists. Data are means $\pm$ S.E.M. from one experiment performed in triplicate, representative of three independent experiments.

D$_2$ receptor contributes to the protection against oxidative-stress-induced apoptosis in PC12-D$_2$R cells.

DISCUSSION

Our results demonstrate that DA agonists can protect PC12 cells that express the human D$_2$ receptor and a nigral DA cell line, SN4741, from apoptosis induced by oxidative stress. Similar protective effects could not be obtained in PC12 cells that lacked D$_2$ receptors. These observations suggest that the D$_2$ receptor plays a critical role in the neuroprotective effects conferred by DA agonists in this model system. Furthermore, we find that specific DA agonists vary in their capacity to provide anti-apoptotic effects and that neuroprotective effects do not correlate closely with the capacity of the agonist to activate classical D$_2$-receptor-coupled G-protein signalling pathways. Furthermore,
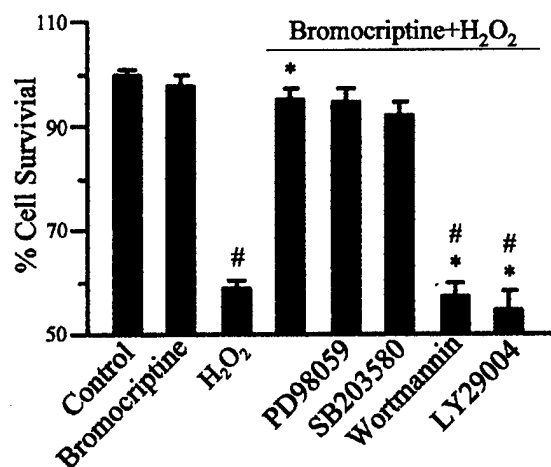
Figure 8   Elimination of DA-agonist-mediated neuroprotection by inhibition of PI 3-kinase

Low concentrations of the PI 3-kinase inhibitor wortmannin (100 nM) or LY294002 (10 μM) eliminate the protective action of bromocriptine on H_2O_2-induced apoptosis. Data plotted are from one experiment (means $\pm$ S.E.M., $n = 8$), representative of four independent experiments. #$P < 0.001$ compared with control (no treatment). *$P < 0.001$ compared with H_2O_2 alone. #*$P < 0.001$ compared with bromocriptine + H_2O_2.

A

B
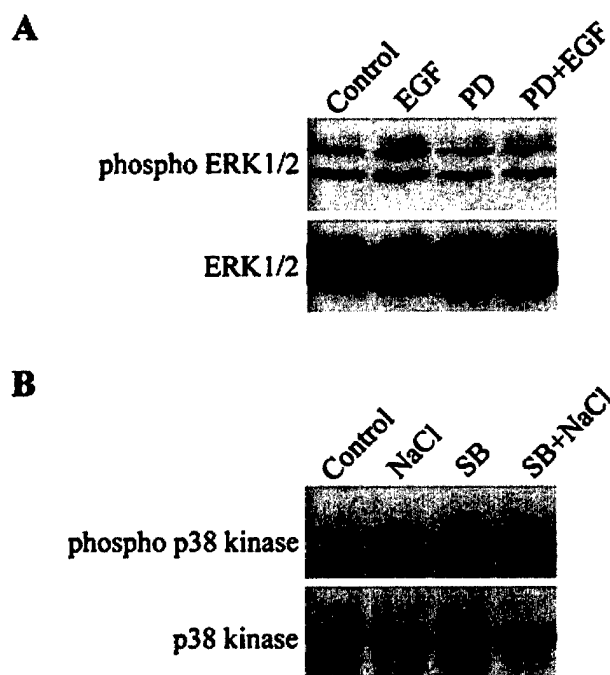
Figure 9   Representative Western immunoblots showing the effect of mitogen-activated protein kinase inhibitors on the phosphorylation of ERK and p38 kinase

(**A**) PC12-D_2R cells untreated (control) or pretreated with 50 μM PD98059 (PD) for 1 h were stimulated with epidermal growth factor (EGF) for 10 min. Western blot analysis was performed using antibodies specific for phosphorylated and total ERK1/2. (**B**) Cells were untreated (control) or pretreated with 20 μM SB203580 (SB) for 1 h and either untreated or treated with NaCl (0.4 M) for 1 h. Western blot analysis was performed using antibodies specific for phosphorylated and total p38 kinase.

we find that activation of the PI 3-kinase signalling pathway is required for anti-apoptotic activity of DA agonists in PC12-D_2R cells.

In our experiments in PC12 cells, we find no evidence for receptor-independent cytoprotective activities of DA agonists. Rather, we find that the activation of DA D_2 receptors is required for the prevention of apoptosis induced by H_2O_2 in undifferentiated PC12 cells and for protection against either H_2O_2 or trophic withdrawal in NGF-differentiated PC12 cells. This conclusion is based on the following observations: (i) no protection was observed in PC12 cells that do not express the D_2 receptor; (ii) the agonists bromocriptine, pergolide and quinpirole all protect PC12 cells from apoptosis in a concentration-dependent manner; (iii) this concentration-dependent protective effect is reversed by D_2 antagonists; and (iv) the promotion of cell survival is agonist-specific and independent of their capacity to activate G-protein coupled signalling pathway. Several previous studies have implicated activation of DA receptors in the neuroprotective effects observed with DA agonists [13,25,26]. For example, the capacity of bromocriptine to protect a mouse HT22 cell line against oxidative stress [26] and rodent dopaminergic neurons from levodopa-induced toxicity [27] is dependent on its ability to stimulate D_4 and D_2 DA receptors respectively. Thus several receptors may activate anti-apoptotic signalling.

Various studies have found that DA agonists can promote cell survival independently of receptor activation in several experimental paradigms [28–30]. Not all reports of DA agonist-mediated protection appear to rely on activation of DA receptors and other mechanisms may be applicable in other model systems. *In vitro* and *in vivo* studies have shown that DA agonists are capable of scavenging superoxide or hydroxyl radicals [30,31]. Pramipexole has been shown to up-regulate Bcl2 expression, which could provide an anti-apoptotic effect [32,33]. In addition, studies in isolated mitochondria have shown that the agonist can protect against membrane swelling induced by calcium or MPP^+, which could not be accounted for by direct receptor activation [34]. In addition, blockade of DA receptors in dopaminergic cell cultures in some studies does not prevent the protective properties of DA agonists [32,35]. Further, the enantiomers of apomorphine and pramipexole, which do not bind to DA receptors, have been reported to protect dopaminergic neurons from MPP^+, H_2O_2 or 6-hydroxydopamine toxicity [36]. These observations indicate that agonists can induce protective effects in some models independent of DA receptors [29,32,35]. These differences with our findings, in which the anti-apoptotic activity of DA agonists in PC12-D_2R cells is predominantly mediated by activation of the D_2 receptors, may result from differences in the experimental systems utilized and/or the DA agonist tested.

DA receptors belong to the rhodopsin family of heptahelical G-protein-coupled receptors. We find that the D_2-receptor-mediated increased cell survival is apparently independent of its activation of G_i/G_o heterotrimeric G-proteins. Recent studies have revealed that, in addition to heterotrimeric G-proteins, these receptors may interact with and activate a variety of signal mediators, including small G-proteins [37], Na^+/H^+-exchange factor [38], c-Src [39] and cGMP-operated Ca^{2+} channels [26]. The differences in their relative neuroprotective efficacy and their activation of G-proteins support the 'agonist signal trafficking' hypothesis that different agonists acting at the same receptor subtype can stabilize distinct receptor conformations and thereby preferentially activate subsets of the signalling pathways coupled to that receptor [40]. A recent study on D_2 receptor–G-protein interactions reported that specific agonists differed in their relative activity at promoting receptor complexing with either G_{i2} or G_o G-proteins, also supporting the formulation that specific agonists can stabilize the D_2 receptor in different conformation [20]. Our data show that specific agonists select between activation of only G_i/G_o G-protein pathways and the additional activation of a G-protein-independent

neuroprotective pathway. This distinction may be important in designing studies to determine the neuroprotective activity of DA agonists *in vivo*.

The present study demonstrates that PI 3-kinase inhibitors block the protective effect of D$_2$-receptor stimulation. Therefore, signal transduction via PI 3-kinase activation is necessary for this protective effect. Although PI 3-kinase is clearly important for growth-factor-mediated neuronal survival in many cell types and conditions, in other neuronal cell types and under different conditions, growth-factor-mediated activation of the ERK-signalling pathway appears to mediate survival effects [41]. We find no evidence for the involvement of ERK in D$_2$-receptor-mediated survival of PC12-D$_2$R cells from our studies using specific inhibitors. It was reported recently that D$_2$-receptor activation protects cortical neurons from glutamate-induced cytotoxicity by up-regulation of Bcl-2 protein expression via the PI 3-kinase cascade [42]. Therefore the effects of D$_2$-receptor activation on PI 3-kinase and cell survival appear to apply to a variety of cellular insults.

We find that a remarkably large effect on survival can be obtained with activation of the D$_2$ receptor by certain agonists. Furthermore, this improvement in survival is quite different with different agonists and does not correlate with the capacity of these agonists to activate classical G-protein signalling. These results suggest that D$_2$ receptors activate a trophic factor linked pro-survival signalling pathway in an agonist-specific manner. Thus it is likely that intracellular signalling by D$_2$-receptor stimulation can be manipulated for the development of more effective neuroprotective therapies.

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Appendix 2

Early single cell bifurcation of pro- and anti-apoptotic states during oxidative stress

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Abstract

In a population of cells undergoing oxidative stress, each individual cell either succumbs to apoptotic cell death or maintains homeostasis and survives. We report that in response to hydrogen peroxide, PC12 cells show both homeostatic responses, as represented by activation of ERK and pro-apoptotic responses as indicated by p53 activation and p53 suppresses ERK activation. Individual cells segregate into two populations within the first hour of stress, either showing the gene induction mediated by activation of ERK or pre-apoptotic p53 activation. Changing the level of oxidative stress alters the relative proportion of pro-apoptotic cells at this early time point. These results provide a mechanistic basis for an early switch-like response to oxidative stress.

Introduction

Oxidative stress has been implicated in the pathophysiology of several human diseases, including atherosclerosis, pulmonary fibrosis, cancer, neurodegenerative disorders and aging[1-3]. Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) are natural by-products generated by living organisms as a consequence of aerobic metabolism[4]. The cellular toxicity of H_2O_2 is initiated by oxidative stress resulting in the rapid modification of cytoplasmic constituents, the depletion of intracellular glutathione and ATP, a decrease in NAD^+ level, an increase in free cytosolic Ca^{2+}, and lipid peroxidation[5]. H_2O_2 also activates the opening of the mitochondrial permeability transition pore and the release of cytochrome c[6]. In the cytoplasm, cytochrome c, in combination with Apaf-1, activates caspase-9 leading to the activation of caspase-3 and subsequent apoptosis[7,8].

Recent emerging evidence demonstrates that ROS are also physiological mediators of cell signaling produced by stimulation with cytokines, peptide growth factors, and receptor agonists[9]. Therefore, ROS can damage various cell components or activate specific physiological signaling pathways, with the relative effects determined by ROS concentration. Oxidative stress activates seemingly contradictory signaling pathways and the consequences of the response vary widely, with the ultimate outcome being dependent on the balance between these stress activated pathways[10,11]. Among the main stress signaling pathways and/or central mediators activated in response to oxidant injury are the extracellular regulated kinase (ERK), c-jun amino-terminal kinase (JNK) and p38 mitogen-

activated protein kinase (MAPK) signaling cascades, the phosphoinositide 3-kinase (PI 3-kinase)/Akt pathway, the nuclear factor (NF) k-B signaling system, p53 activation, and the heat shock response (see review[11]). Activation of these pathways is not unique to oxidative stress, as they are known to have central roles in regulating cellular responses to other stresses as well as regulating normal growth and metabolism. In general, the heat shock response, ERK, PI 3-kinase/Akt and (NF) k-B signaling pathways exert a pro-survival influence during oxidant injury[10,12-17], whereas, activation of p53, JNK and p38 are implicated in apoptosis [10,18-23].

The initiating events leading to activation of these different signaling pathways in response to oxidants are incompletely understood. Oxidative stress is known to induce apoptosis in a dose-response manner[13,17,24]. When exposed to a level of oxidative stress that can induce apoptosis in some proportion of cells, each individual cell must proceed through a decision-making process that ultimately results in either survival or death. Defining the time-course over which this cell-fate bifurcation occurs is essential for understanding the decision-making mechanisms and for designing molecular strategies for intervening in the process. We find that early after H_2O_2 exposure, each cell activates either homeostatic or pre-apoptotic signaling pathways, but not both.

Results

ERK and p53 pathways are activated in response to oxidative stress

PC12-D$_2$R cells undergo apoptosis when exposed to H$_2$O$_2$ in a concentration and time dependent manner[24]. Multiple signal transduction pathways are activated in response to oxidative stress [11]. To identify the signaling mechanisms activated in response to H$_2$O$_2$, we have assessed the activation of ERK, JNK, p38-kinase, and p53, using Western blot analysis with antisera against phospho-ERK, phospho-JNK, and phospho-p38 kinase, we found that 200 µM H$_2$O$_2$ rapidly induced the phosphorylation of ERK, but not of JNK or p38 kinases in PC12-D2R cells (Fig. 1a, b and c). The activation of ERK by H$_2$O$_2$ was rapid and sustained. (Fig. 1a, top). Anti-phospho-Ser15 antibody was used to detect DNA-damage-induced phosphorylation of p53[25,26] following exposure to H$_2$O$_2$ up to 6 h. In PC12-D$_2$R cells, p53 phosphorylation was significantly enhanced within 30 min following H$_2$O$_2$ exposure and continued to increase for up to 2 h (Fig. 1d). The level o f p53 protein was remained unchanged for 6 h incubation with H$_2$O$_2$ (Fig. 1d). These results demonstrate the early activation of ERK and p53 signaling pathways in response to oxidative stress in PC12-D$_2$R cells.

Activation of p53 in response to oxidative stress down-regulates ERK

Our results demonstrated that H$_2$O$_2$ activates both ERK and p53 in PC12-D$_2$R cells. To study the cross talk between ERK and p53 signaling mechanisms, we used the selective inhibitor of ERK, PD98059 to block phosphorylation of ERK[27]. In Western immunoblots using phospho-ERK and phospho-p53 antibodies, it was determined that addition of

PD98059 (100 µM) 1 h prior to H_2O_2 treatment prevented the phosphorylation of ERK (Fig. 2a). However, PD98059 did not affect the activation of p53 (Fig. 2a).

To examine the effect of p53 on the activation of ERK, we used the p53 inhibitor, pifithrin alpha (PFT)[28]. In Western immunoblots using phospho-ERK and phospho-p53 antibodies, we have found that PFT augmented the activation of ERK in presence of H_2O_2 (Fig. 2b). Consistent with the effect of PFT on p53 phopshorylation (ref) we found 40 µM of PFT had no effect on the phosphorylation of p53 in response to H_2O_2. These results suggest that H_2O_2-induced ERK phosphorylation is negatively regulated by activation of p53.

Characterization of ERK activated gene program in response to oxidative stress

To identify the cellular response during oxidative stress-induced apoptosis, oligonucleotide microarrays were used to analyze the gene expression profile associated with H_2O_2 exposure and regulated transcripts were confirmed by quantitative real-time PCR analysis (QPCR). As shown in Table1, genes that encode transcription factors including *egr1, c-fos, c-jun, pc3*, and a zinc finger protein were up-regulated after 200 µM H_2O_2 for 1 h. Other significantly up-regulated genes include inner mitochondrial membrane component, ATP synthase subunit c, and stress response gene, *hsp70*, the immediate-early inducible small GTP binding protein, *rhoB*, and a gene of unknown function. We also found that the MAP kinase phosphatase-1 (*mkp1*) was significantly increased by >3 fold in cells treated with H_2O_2. The data indicated that oxidative stress activates signaling pathways and induce multiple target genes. It is known that activation

of ERK-dependent transcriptional activation events are mediated by activation of transcription factors such as c-fos[13], egr1[29] pc3[30] and mkp1[31]. Stress has been known to induce rhoB[32] and heat shock protein 70 (hsp70)[12].

To identify the component of the oxidative stress-induced gene program downstream of ERK activation, we inhibited ERK with PD98059. Addition of PD98059 (100 μM) 1 h prior to H_2O_2 treatment decreased the induction of egr1, pc3, and mkp1 (Fig. 3). Egr1 was originally identified as an immediate early gene induced by ERK in response to NGF in PC12 cells[33]. PD98059 did not prevent the induction of c-fos, copeb, c-jun, hsp70, rhoB, ATP synthase subunit c, and EST (AI639167).

Characterization of egr1 induction in response to oxidative stress

In order to understand the mechanism underlying this ERK-dependent signaling pathway in response to H_2O_2 at the single cell level, we studied the induction of egr1 mRNA using fluorescent in situ hybridization (FISH). When cells were exposed to 200 μM of H_2O_2, we observed approximately half of the cells with strong fluorescent signal (Fig. 4). The signal in uninduced cells was indistinguishable from that of control cells. Cells hybridized with sense-oriented probe for egr1 showed no fluorescent signal in any cells (data not shown). Simultaneous double-FISH for egr1 and β-actin mRNA showed no change in β-actin mRNA expression during H_2O_2 treatment (Fig. 4). These data demonstrate that H_2O_2 induces a high level of egr1 induction in a subset of cells and no detectable change in egr1 levels in others.

ERK-mediated egr1 induction is independent of p53 activation

Signaling through ERK is known to be pro-survival[10,13,15,34] and the p53 activation

is known to be pro-apoptotic[18,19,23]. The p53 protein plays a central role in the cellular

response to DNA damage[35,36] that leads to phosphorylation and activation of p53[37-39]. To

characterize signaling pathways activated by ROS, we monitored *egr1* expression and

ERK or p53 signaling pathways following H_2O_2 exposure. Nuclear translocation of

activated ERK and p53 is thought to be critical for transcriptional regulation of responsive

genes[40,41]. Thus we followed the intracellular movement of activated ERK and p53.

Immunocytochemical staining of PC12-D_2R cells using a phospho-ERK or phospho-p53

specific anti-serum revealed marked nuclear transport in response to H_2O_2. Enhanced

phospho-ERK protein levels were detected both in the cytoplasm and in the nucleus (Fig.

5a, top). However, phospho-p53 was mainly localized in the nucleus (Fig. 5a, bottom).

Approximately half of the cells showed ERK or p53 phosphorylation in response to 200

µM of H_2O_2 (Fig. 5b).

We next used double-labeling to study whether there was overlap of p53 and

pERK/*egr1* induction within the same cells. As shown in Fig. 6a, *egr1* mRNA co-localized

to cells showing ERK activation. However, the induction *egr1* mRNA by H_2O_2 was absent

in p53 activated cells (Fig. 6b). These results suggest that oxidative stress activates ERK or

p53 signaling pathways in separate cell sub-populations.

Activation of ERK and p53 is dependent on the concentration of H_2O_2

We have demonstrated that 200 μM of H_2O_2 activates ERK or p53 and are localized in separate cell sub-populations. To elucidate the role of these pathways in cell survival or death, we examined the effect of varying concentrations of H_2O_2 on ERK and p53 activation. PC12-D_2R cells undergo concentration dependent apoptosis when exposed to H_2O_2[24]. As shown in Fig. 7, at low sub-lethal concentrations of H_2O_2 activated ERK but not p53. At higher concentrations of H_2O_2 (200-400 μM) both ERK and p53 phosphorylation are observed. At the single cell level, 100 μM of H_2O_2 phosphorylated ERK/*egr1* in almost all the cells. However, 200 μM of H_2O_2 activated ERK/*egr1* in only half of the cells (Fig. 7b).

Caspase-3 activation occurs in cells not showing ERK activation in response to oxidative stress.

Double immunostaining for phospho-ERK and phospho-p53 showed p53 and ERK are activated in different cells in the presence of 200 μM of H_2O_2 for 1h (Fig. 8a). When observed at 24 h, the condensed nuclei and active casapase-3, which are hallmarks of apoptosis, are found exclusively in phospho-ERK negative cells (Fig. 8b). These data indicate that by 1h the cells have segregated into two populations: those that activates p53 and will proceeds to apoptosis and that those activate ERK/*egr1* will maintain homeostasis.

Discussion

In this study we demonstrate that when cells are exposed to oxidative injury they activate specific signaling pathways within the first half hour that indicate whether those cells will ultimately tolerate or succumb to the insult. ERK activation marks cells that choose, early in oxidative stress to maintain homeostasis [13]. Oxidative stress also produces genotoxic damage[35,36] that results in activation of p53 leading to cell death[42]. Although the molecular events leading to the activation of these signaling pathways have not been defined, our data indicate that ROS-mediated anti- and pro-apoptotic signaling events are triggered very early on following exposure to oxidative stress, are sustained and are mutually exclusive.

We find that activation of ERK and *egr1* within 1h, mark cells destined to survive after initial oxidative insult. In PC12 cells, ERK is mainly activated by growth factors and has been shown to be associated with cell proliferation, differentiation, and promotion of cell survival[10,43]. Addition of nerve growth factor to PC12 cells induces various cellular responses, including ERK activation and the induction of immediate early gene *egr1*[29,33]. Evidence for an anti-apoptotic role for ERK has been reported in PC12 cells after growth factor withdrawal[10]. ERK has also been reported to function as a suppressor of ROS in superior cervical ganglion neurons[44]. Activation of the ERK MAPKs via the Ras/Raf/MEK pathway supports peripheral and central neuron cell survival[14,15,34].

We find that the early activation of p53 by H_2O_2 predicts the later induction of capsase-3 and apoptosis following H_2O_2 exposure. The inability of sub-lethal dose of H_2O_2

to activate p53, further supports the involvement of p53 in apoptosis in these cells. Cells exposed to ROS cause nuclear DNA double-stranded breaks that are detected by enzymes from the PI 3-kinase family[45], resulting in phosphorylation of serine 15 of p53[25,26] its consequent stabilization and accumulation[37]. Abnormal p53 activation is implicated in apoptosis in many experimental systems[46]. Activation of p53 results in the up-regulation of proteins implicated in cell cycle control and apoptosis[47] such as proapoptotic BAX and caspase-3[19,23,48].

We demonstrate the activation of two signaling pathways in response ROS that are closely associated with cell survival or apoptosis. It has been reported that, cisplatin-induced ERK activation is an up-stream regulator of the p53 response to DNA damage caused by cisplatin in ovarian carcinoma cell line A2780 [49]. However, in our experimental system, the pharmacological inhibition of ERK had no effect on the phosphorylation p53 in response to H_2O_2. However, the attenuation of ERK activation by p53 inhibition clearly indicates that oxidative stress-induced ERK activation in PC12 cells functions as an anti-apoptotic signal independently of its ability to regulate p53 phosphorylation and caspase-3 activation. The existence of positive and negative signals leading from p53 to ERK might be important for the signal intensity as a major determinant of the cellular response to ROS.

It has been hypothesized that ROS activate stress signaling pathways and that the dynamic balance between pathways may be important in determining whether a cell survives or undergoes apoptosis (see ref[11]). Our results suggest that biochemical assays may obscure the actual mechanisms by failing to differentiate responses that are segregated

within different populations of cells. Our data show that and that even at early time points these competing pathways do not coexist within the same cell. We suggest a new model, analogous to the divergence of cell fate during development, in which each individual cell rapidly respond to stress and achieves a coherent physiological state directed towards either apoptosis or survival. The observation that the proportion of cells recruited into each state depends on the concentration of H_2O_2 suggests a stochastic decision making process. The presence of discrete cellular states early after a toxic stimulus needs to be considered in devising therapeutic strategies to promote cell survival in apoptosis-related human diseases.

Methods

U34 array gene chips were from Affymetrix (Santa Clara, CA). Antibodies specific to phospho-ERK, ERK, phospho-p53, p53, phospho-JNK, JNK, phospho-p38kinase and p38 kinase were from Cell Signaling Technology (Beverly, MA). Anti-active caspase-3 antibody was from Promega (Madison, WI). Alexa fluor 488, goat anti-mouse alexa fluor 568 and goat anti-rabbit alexa fluor 488 conjugated secondary antibodies were from Molecular Probes (Eugene, OR). CY3 was from Clontech (Palo Alto, CA). Donkey anti-rabbit CY3 was from Jackson ImmunoResearch (West Grove, PA). In vitro transcription kit was from Ambion (Austin, TX). Pifithrin alpha and PD98059 were from A.G. Scientific (San Diego, CA) and Calbiochem (La Jolla, CA), respectively.

Cell culture

PC12-D_2R^{24} cells were maintained in DMEM supplemented with 500 μg/ml G418 (Life Technologies, Gaithersburgh, MD), 10% horse and 5% fetal bovine serum (GIBCO) in a humidified atmosphere containing 5% CO_2 at 37°C.

Immunoblotting

Cells were washed twice with ice-cold PBS and lysed in buffer 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Igepal C630, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovandate, 5 μg/ml aprotinin and cocktail of protease inhibitors (Roche Diagnostics, GmbH) at 4°C for 20 min. After centrifugation at 14,000 x g for 20 min at 4°C, equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis. The

resolved proteins were electrotransferred to nitrocellulose membranes. Detection of proteins by immunoblotting was conducted using ECL system according to the manufacturer's recommendations. The blots were then stripped in buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS and 100 mM β-mercaptoethanol for 30 min at 50°C and re-probed with respective antibodies.

Gene expression analysis

Total RNA was isolated from PC12-D$_2$R cells with the Stratagene (LaJolla, CA) StrataPrep total RNA miniprep kit according to the manufacturer's protocol. Microarray analysis was performed by using an oligonucleotide gene chip, Rat Genome U34 array (Affymetrix, Santa Clara, CA) as previously described[50]. Total RNA was isolated from PC12-D$_2$R cells treated with 200 μM of H$_2$O$_2$ for 1 h and used for microarray experiments. Affymetrix microarray suite 5.0 was used to analyze the raw data using the criteria of 60% concordance across multiple array comparison's and fold changes greater than or equal to 1.6 for outlier detection. Quantitative real-time PCR was carried out in an ABI Prism 7900HT (Applied Biosystems, Foster City, CA) as described[50]. Amplicon size and reaction specificity was confirmed by agarose gel electrophoresis. The results were normalized to *β-actin* and expressed as the fold of H$_2$O$_2$ over control values.

Immunocytochemistry

PC12-D$_2$R cells growing on collagen coated coated cover glass were treated as indicated. The cells were fixed, permeabilized as described[24] and immunocytochemical staining for phospho-ERK, phospho-p53, or active caspase-3 was carried out. Anti-phospho-ERK (dil.

1:400), anti-phospho-p53 (dil. 1:500), or active caspase-3 antibody (dil. 1:200) was added and incubated overnight at 4°C. Following washing, cells were incubated with corresponding secondary antibodies for 2h at room temperature. The cells were washed twice in PBS and the nuclei were stained with 1 μg/ml (in PBS) of the fluorescent DNA dye DAPI (Sigma) for 10 min and then washed with PBS. The liquid was drained and the slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) mounting medium.

Fluorescent *in situ* hybridization

Egr1 (162bp) and *β-actin* (150 bp) were amplified from RNA isolated from PC12-D$_2$R cells by PCR. The DNA fragments were purified from agarose gel using QIAEX II gel extraction kit (Stratagene) and sub-cloned in to pDrive vector (Qiagen, Valencia, CA). Individual clones were sequenced to determine the orientation of the DNA. To generate cRNA probes, we used T7 promoter for anti-sense and SP6 promoter for sense probes to minimize the interference of vector sequences in double *in situ* hybridization. Aminoallyl-UTP (Ambion, Austin, Texas) incorporated cRNA probes were generated using a commercial transcription kit (Maxiscript, Ambion). The yield and integrity of riboprobes was confirmed by gel electrophoresis. The *egr1* and *β-actin* cRNA probes were labeled with Alexa Fluor 488 and CY3, respectively, according to manufacturer's protocols. Riboprobes were purified on Atlas nucleospin columns (Clontech).

To visualize the mRNA of interest, fluorescent *in situ* hybridization was performed. The cells grown on cover slips were fixed and permeabilized as described[24]. Following

prehybridization in 5X SSC, 50% formamide, and 1 mg/ml tRNA at room temperature for 30 min, the denatured probe was added to the prehybridization buffer. Hybridization was carried out for 2 h at 52°C. Following two 5-min washes in 5X SSC, 50% formamide, 0.1% SDS and twice in 2X SSC, the nuclei were stained with DAPI and slides were mounted in Vectashield mounting medium. When immunostaining was carried out following hybridization the slides were incubated with respective antibodies as described above.

Figure Legends

Figure 1 **Overall response of signal transduction pathways to oxidative stress.** *a*, Oxidative stress significantly increased ERK phosphorylation within 30 min after the addition of H_2O_2. *b* and *c*, H_2O_2 showed no effect on the phosphorylation of JNK and p38 kinase, respectively. *d*, H_2O_2 significantly increased p53 phosphorylation within 30 min after the addition of H_2O_2. The PC12-D$_2$R cells were incubated with 200 μM of H_2O_2 for indicated period of time and Western immunoblots were carried out using respective antibodies. The blots were stripped and reprobed using the antibodies recognizing the total ERK, JNK, p38 kinase or p53 proteins. The experiments were repeated four times with similar results.

Figure 2 **Cross talk between p53 and ERK in response to oxidative stress.** *a*, The cells were pretreated with or without 100 μM PD98059 for 1 h and incubated with 200 μM H_2O_2 for 1 h. *b*, The cells were pretreated with or without 40 μM of PFT for 1 h and incubated with 200 μM H_2O_2 for 1 h. Aliquots of cell extracts were then subjected to immunoblot analysis using antibodies against the active forms of ERK or p53. The blots were stripped and reprobed using the antibodies recognizing the total ERK or p53 proteins. The experiments were repeated three times with similar results.

Figure 3 **Effect of ERK inhibition on oxidative stress activated gene program.** Total RNA was isolated from PC12-D2R cells untreated or treated with 200 μM of H_2O_2 for 1h in the presence or absence of 100 μM of PD980059 (n=5). The changes in gene expression measured using quantitative RT-PCR analysis of RNA from control and treated cells. The

primer sequences used for RT-PCR were presented in supplemental data. The results were normalized to *β-actin* and expressed as the fold of H_2O_2 over control values. All reactions were performed in triplicate and the resulting mean±s.e.m. values are given. The graph represents one experiment repeated three times essentially with same results. *p<0.001 compared with control. #p<0.001 compared with H_2O_2

Figure 4. *Egr1* **mRNA induction in response to H_2O_2.** *a*, Double-label FISH for *egr1* mRNA (green) and *β-actin* mRNA (red) of control- and PC12-D_2R cell exposed to 200 μM H_2O_2 for 1h. *b*, Percentage of PC12-D_2R cells showing *egr1* mRNA induction after exposure to H_2O_2 for 1 h. The mean±s.e.m. The percentage of egr1 induction was 51±5 % of a total of ~1800 cells scored in four experiments.

Figure 5 **ERK and p53 activation in response to H_2O_2.** *a*, Immunofluorescence microscopy of PC12-D_2R cells treated or untreated with H_2O_2 (200 μM) for 1 h. Cells were labeled for phosphorylated ERK (red, upper panels) or phosphorylated p53 (red, bottom panels). The nuclei were stained with DAPI (blue). *b*, Percentage of PC12-D_2R cells showing phospho-ERK or phospho-p53 in response to H_2O_2 (200 μM) for 1 h. Cells showing fluorescence phospho-ERK or phospho-p53 antibodies was scored as p-ERK or p-p53, respectively (n=5). Values represent the mean±s.e.m. (200-400 cells scored per experiment).

Figure 6 **Oxidative stress activates ERK and p53 in different populations of cells.** *a*, FISH-immunofluorescence microscopy of PC12-D_2R cells labeled with *egr1* mRNA (green) and anti-phospho-ERK (red). *b*, PC12-D_2R cells labeled with *egr1* mRNA (green)

and anti-phospho-p53 (red). Cells were incubated with 200 µM of H_2O_2 for 1h. The experiments were repeated four times with similar results.

Figure 7   Dose response of ERK and p53 in PC12-D₂R to H_2O_2. *a*, The cells were treated with the concentrations of H_2O_2 indicated for 1h. Aliquots of cell extracts were then subjected to immunoblot analysis using antibodies against the active forms of ERK or p53. The experiments were repeated three times with similar results. *b*, Concentration-dependent activation of ERK at single cell level. Immunofluorescene microscopy of active-ERK (red) in control, cells treated with 100 µM or 200 µM of H_2O_2 for 1h. *c*, Concentration-dependent induction of *egr1* at single cell level. FISH for *egr1* (green) in control, cells treated with 100 µM or 200 µM of H_2O_2 for 1h. The nuclei were stained with DAPI (blue). The experiments were repeated three times with similar results.

Figure 8   p53 activation correlates with caspase-3 mediated apoptosis. *a*, Double-labeling for phospho-ERK (red) and phopsho-p53 (green) in PC12-D₂R cells incubated with 200 µM of H_2O_2 1 h. *Arrowheads* indicate cells with activated ERK and no phospho-p53. *Arrows* identify cells with phosphorylated-p53 (green) with no phospho-ERK. *b*, double-labeling of phosphorylated-ERK (red) and active-caspase3 (green) in PC12-D₂R cells incubated with 200 µM of H_2O_2 for 24 h. *Arrows* indicates apoptotic cells with condensed nuclei. Active caspase-3 was localized exclusively in cells with condensed nuclei that are phospho-ERK negative. The experiments were repeated three times with similar results.

Tables

Table 1. *Effect of H_2O_2 on gene expression in PC12 cells*

Identifier	Gene description	Fold Change	
		Microarray	QRT-PCR
AF023087	Nerve growth factor induced factor A (*egr1*)	5.66 ± 1.73	25.01 ± 1.12
M18416	„	4.25 ± 2.18	
U75397	„	3.78 ± 6.23	
S81478	Oxidative stress-inducible protein tyrosine phosphatase (*mkp1*)	4.12 ± 5.95	5.47 ± 1.12
AA945867	*c-jun*	4.12 ± 3.20	1.93 ± 0.30
AI175959	„	2.58 ± 0.58	
M60921	NGF-inducible anti-proliferative putative secreted protein (*pc3*)	3.32 ± 3.70	5.50 ± 0.70
M60921	„	1.63 ± 0.26	
AA944156	„	1.58 ± 0.40	
AF001417	Core promoter element binding protein (*copeb*)	2.76 ± 1.99	2.18 ± 0.50
AI639167	EST	2.33 ± 1.41	1.76 ± 0.42
D13123	P1 mRNA for ATP synthase subunit c	1.97 ± 0.54	1.70 ± 0.50
X06769	*c-fos*	1.92 ± 0.41	2.00 ± 0.70
AA900505	*rhoB*	1.83 ± 0.19	1.78 ± 0.60
L16764	Heat shock protein 70 (*hsp70*)	1.60 ± 0.41	1.45 ± 0.50

Total RNA was isolated from PC12-D_2R cells untreated or treated with 200 µM of H_2O_2
for 1h. Changes in the expression level of H_2O_2 induced genes in Affimetrix analysis (n=4)
were independently confirmed using quantitative RT-PCR (QRT-PCR) analysis (n=5) of
RNA from control and H_2O_2 treated cells. The results were normalized to *β-actin* and
expressed as the fold of H_2O_2 over control values. All reactions were performed in
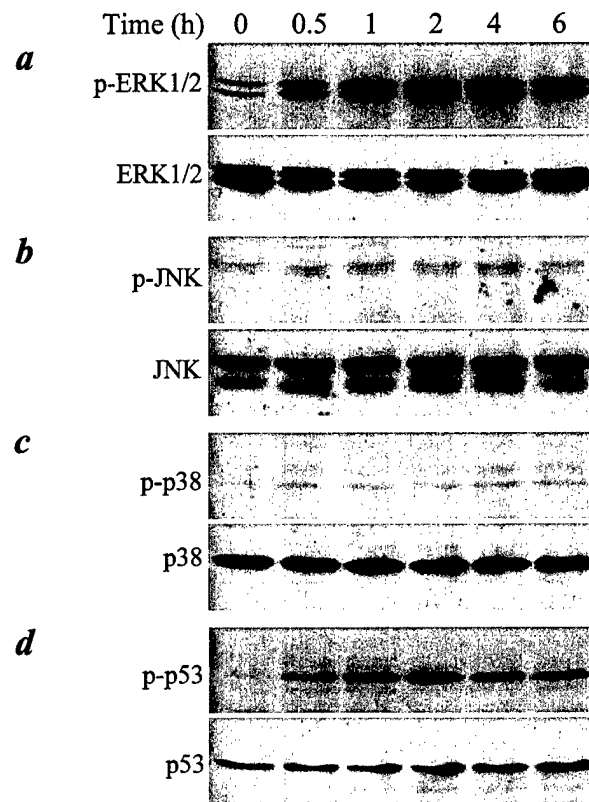triplicate from two different experiments, and the resulting s.e.m. values are also given.

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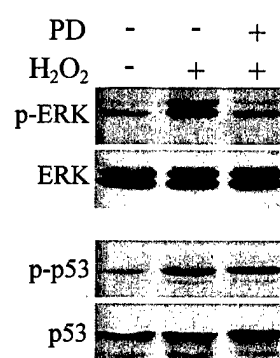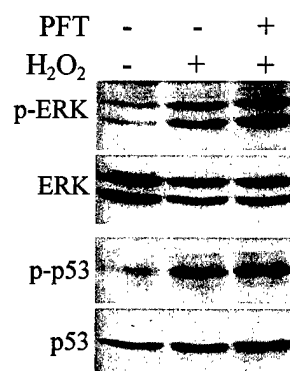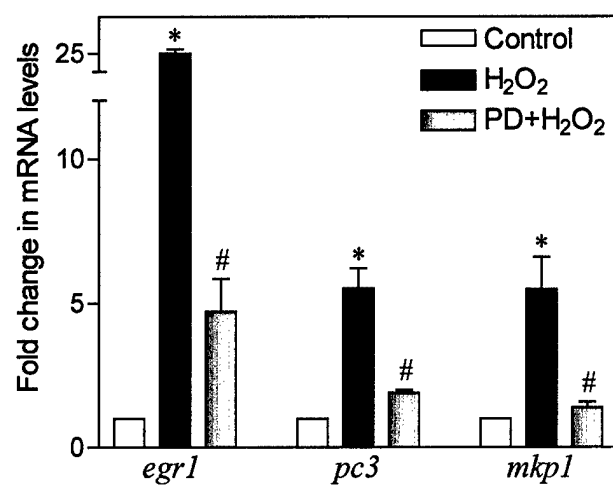
Figure 1

Figure 2

a
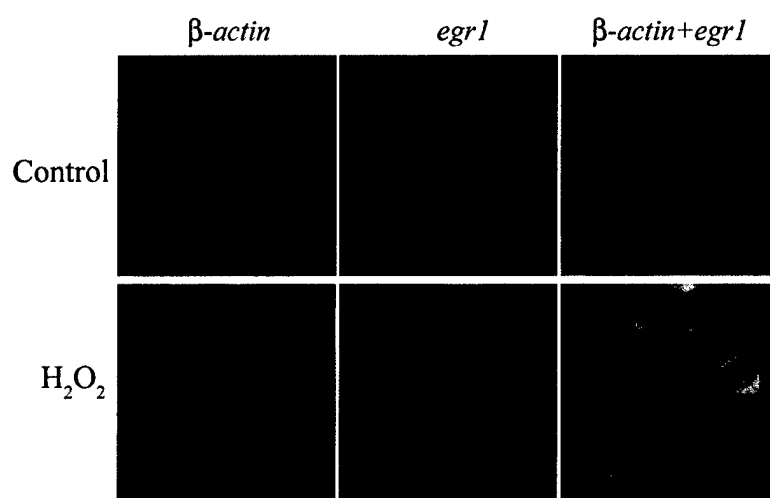
PD   −   −   +
H_2O_2 −   +   +

p-ERK

ERK

p-p53

p53

b

PFT   −   −   +
H_2O_2 −   +   +

p-ERK

ERK

p-p53

p53

Figure 3

Figure 4

	β-*actin*	*egr1*	β-*actin+egr1*
Control			
H₂O₂			

Figure 5

a

Control   H₂O₂

p-ERK

p-p53

b

Figure 6

a   *egr1*   p-ERK   *egr1*+p-ERK

b   *egr1*   p-p53   *egr1*+p-p53

Figure 7

a   H$_2$O$_2$ (µM)   0   50   100   200   400

p-ERK1/2

p-p53

b

	Control	H$_2$O$_2$ (100 µM)	H$_2$O$_2$ (200 µM)

p-ERK

c

egr1

Figure 8

a

	p-ERK	p-p53	p-ERK+p-p53

H₂O₂
(1 h)

b

	p-ERK	Active caspase-3	p-ERK+ Active caspase-3

H₂O₂
(24 h)
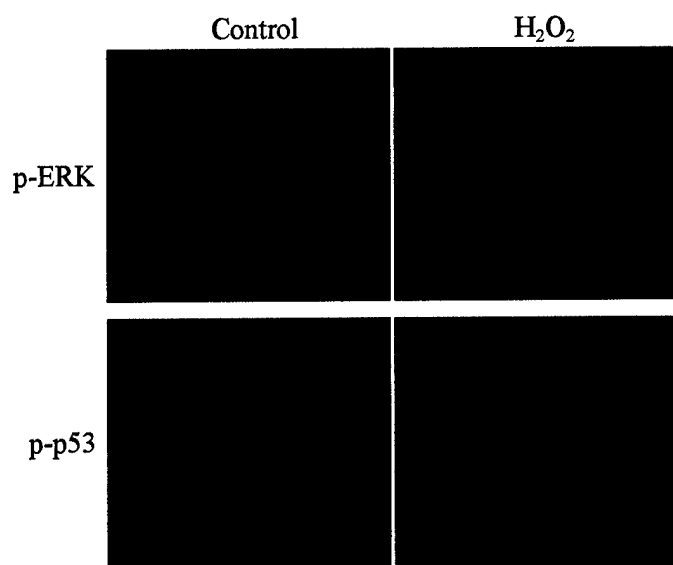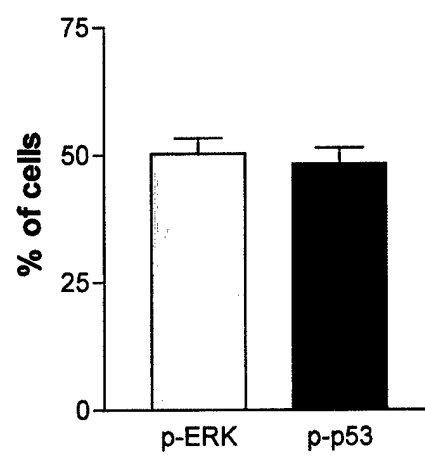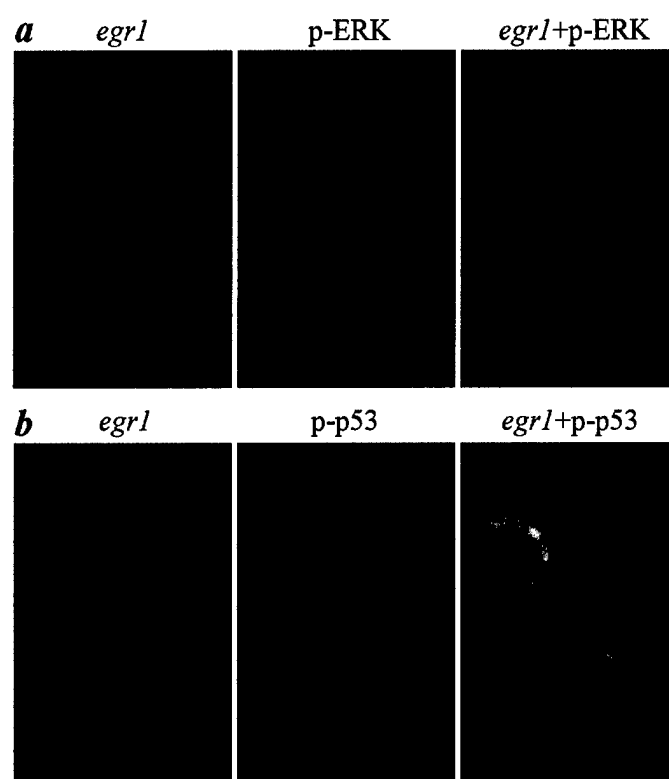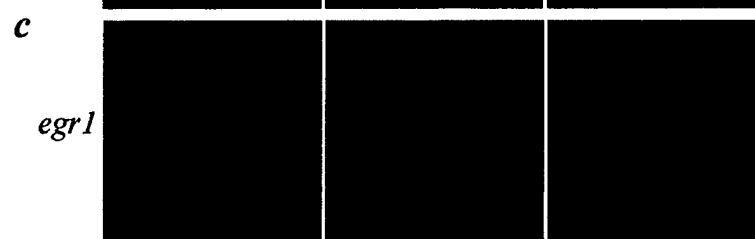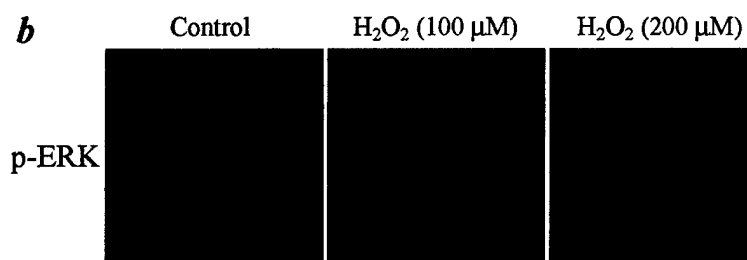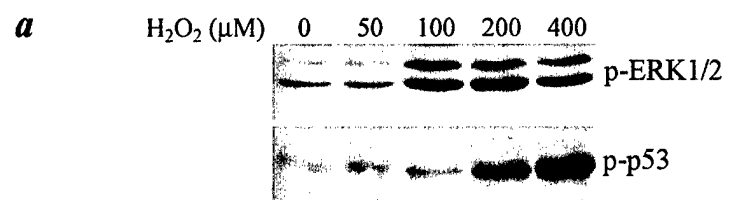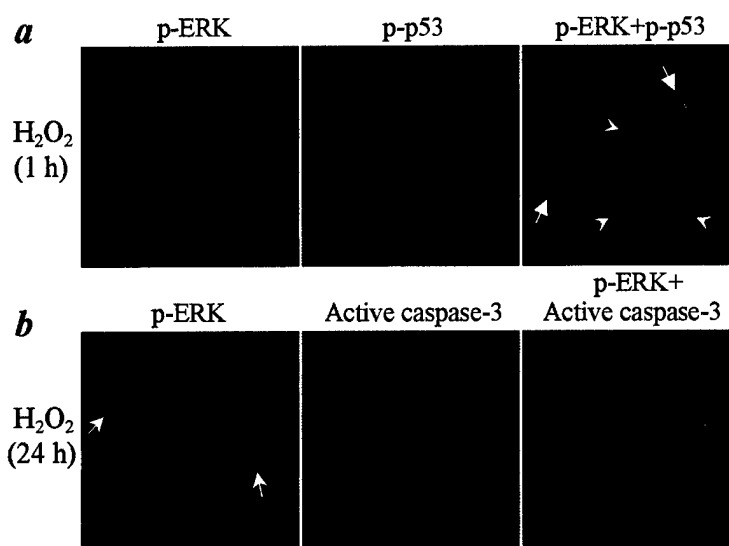
Appendix 3

Agonist Specific Transactivation of Phosphoinositide 3-kinase Signaling Pathway Mediated by the Dopamine D$_2$ Receptor

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Running Title: D$_2$ receptor signaling crosstalk

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SUMMARY

Bromocriptine, acting through the dopamine D_2 receptor, provides robust protection against apoptosis induced by oxidative stress in PC12-D_2R and immortalized nigral dopamine cells. We now report the characterization of the D_2 receptor signaling pathways mediating the cytoprotection. Bromocriptine caused protein kinase B (Akt) activation in PC12-D_2R cells and the inhibition of either phosphoinositide 3-kinase (PI 3-kinase), epidermal growth factor receptor (EGFR) or c-Src eliminated the Akt activation and the cytoprotective effects of bromocriptine against oxidative stress. Co-immunoprecipitation studies showed that activation of the D_2 receptor induced its association with the EGFR, suggesting a cross-talk between these receptors in mediating the activation of Akt. EGFR repression by inhibitor or by RNA interference, eliminated the activation of Akt by bromocriptine. D_2 receptor stimulation by bromocriptine induced c-Src tyrosine 418 phosphorylation and EGFR phosphoryation specifically at tyrosine 845, a known substrate of Src kinase. Furthermore, Src tyrosine kinase inhibitor or dominant negative Src interfered with Akt translocation and phosphorylation. Thus, the predominant signaling cascade mediating cytoprotection by the D_2 receptor involves c-Src/EGFR transactivation by D_2 receptor, activating PI 3-kinase and Akt. We also found that the agonist pramipexole failed to stimulate activation of Akt in PC12-D_2R cells, providing an explanation for our previous observations that, despite efficiently activating G-protein signaling, this agonist had little cytoprotective activity in this experimental system. These results support the hypothesis that specific dopamine agonists stabilize distinct conformations of the D_2 receptor that differ in their coupling to G proteins and to a cytoprotective EGFR-mediated PI-3 kinase/Akt pathway.

INTRODUCTION

The hallmark of Parkinson's disease (PD) is the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (1), causing a profound reduction in dopamine-mediated signaling (2). The prominent locomotor deficits that occur in this disease are in large part attributable to the loss of stimulation of the dopamine D_2 receptor (3), a member of the rhodopsin-like heptahelical receptor family (4). The D_2 receptor is an important target for antiparkinsonian drugs that ameliorate the motor deficits associated with this disorder. In recent years, dopamine agonists have also been found to have neuroprotective activity in some experimental models, and the possibility that they may decrease the progression of PD has been proposed (5). However, the mechanisms underlying the agonist-mediated neuroprotection reported in experimental models are poorly understood and the potential for dopamine agonists to alter the clinical course of this disease remains an area of controversy (6).

Many heptahelical receptors couple to multiple signal transduction pathways, including various heterotrimeric G-protein-second messenger pathways and growth factor receptor-protein kinase cascades (7). The signal for activation of the proximal mediators of signaling such as heterotrimeric G-proteins, receptor kinases or other protein partners, is an alteration in the receptor's conformation that occurs following complexing with agonist. Studies in several

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[1]*Abbreviations used*: PD, Parkinson's disease; H_2O_2, hydrogen peroxide; PI 3-kinase, phosphoinositide 3-kinase; Akt, protein kinase B; PH, pleckstrin homology; GFP, green fluorescent protein; EGFR, epidermal growth factor receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; NGF, nerve growth factor; PTX, pertussis toxin; ECL, enhanced chemiluminescence lighting; PIP3, phosphatidylinositol 3,4,5-triphosphate; PDGF, platelet-derived growth factor; siRNA, small interfering RNA, GPCR, G protein-coupled receptor

heptahelical receptors suggest that these proteins exist in multiple, functionally significant conformations that may differ in their relative activation of different signaling pathways (8-12). Studies with several receptors, including the dopamine D_2 receptor, suggest that agonists acting at the same receptor select among different active receptor conformations and determine the relative levels of activation of downstream signaling pathways, a hypothesis called agonist-directed signal trafficking (13-16).

We had previously investigated the role of the D_2 receptor expressed in the PC12 cell line (PC12-D_2R) in modulating the induction of apoptotic cell loss caused by hydrogen peroxide-induced (H_2O_2) oxidative stress (17). Although the mechanism of neuronal loss in PD is not known, many studies have implicated oxidative stress (reviewed in Refs. (18,19). Oxidation of dopamine by auto-oxidation and monoamine oxidase produces reactive oxygen species including H_2O_2. H_2O_2 reacts with ferrous (Fe_2^+) iron to produce hydroxyl radicals, which can damage proteins, nucleic acids, and membrane phospholipids, and induce apoptosis (20). Some animal model and human PD postmortem studies provide evidence that the degeneration of DA neurons occurs via apoptosis (21,22). We found that activation of the D_2 receptor in the PC12-D_2R line caused a robust, concentration dependent increase in cell survival during oxidative stress that required activation of phosphoinositide 3-kinase (PI 3-kinase). Among the agonists studied, we found significant discrepancies in the capacity of individual agonists to mediate anti-apoptosis and to stimulate G-protein activation, assayed via [^{35}S]GTPγS binding (17).

In order to elucidate the mechanisms underlying agonist-specific modulation of cell survival, we have now investigated the anti-apoptotic signaling pathway activated by the D_2 receptor. We find that D_2 receptor-mediated protection against oxidative stress involves a novel

c-Src-dependent transactivation of the epidermal growth factor receptor (EGFR) that activates PI 3-kinase/protein kinase B (Akt) and that agonists differ in their capacity to activate this pathway.

EXPERIMENTAL PROCEDURES

Chemicals - (+)-Bromocriptine methanesulfonate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), H_2O_2, nerve growth factor (NGF) and pertussis toxin (PTX) were purchased from Sigma Chemicals (St. Louis, MO). Pramipexole was a gift from Pharmacia (Kalamazo, MI). Lipofectamine, DMEM, and fetal calf serum were obtained from Life Technologies (Gaithersburgh, MD). AG1478, AG1296, k252a, PP2, LY294002, and wortmannin were obtained from Calbiochem (La Jolla, CA). Epidermal growth factor (EGF) was obtained from Invitrogen (Carlsbad, CA). Enhanced chemiluminescence lighting (ECL) Western blotting detection reagent kit was from Amersham Pharmacia Biotech (Piscataway, NJ). Antibodies specific to phospho-Akt, Akt, phospho-tyrosine EGFR, EGFR were from Cell Signaling Technology (Beverly, MA). Anti-phospho-c-Src and c-Src antibodies were from Biosource International Inc. (Camarillo, CA). Mouse monoclonal antibodies to dopamine D_2 receptor, phospho-tyrosine (PY20) and protein A/G agarose were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and Viability analysis - The development and characterization of PC12-D_2R cell line, which are stably transfected with the human D_{2L} receptors were previously described (17). The cells were maintained in DMEM supplemented with 10% horse serum, 5% fetal bovine serum and 500 µg/ml G418 in a humidified atmosphere containing 5% CO_2 at 37°C. For differentiation, PC12-D_2R cells were plated onto collagen-coated plates in DMEM containing 10% horse serum and 5% fetal bovine serum and allowed to attach overnight. The cells were then induced to differentiate by growing in DMEM supplemented with 1% fetal bovine serum and

100 ng/ml NGF for 10-14 days. Nigral dopamine cell line SN4741 (generous gift from Dr. J. H. Son Columbia University, New York) was cultured as described (23). Cell viability was measured by the MTT method 24 h after various treatments as described (17).

Transfections and DNA constructs - For live cell fluorescent microscopy, PC12-D_2R cells (1 x 10^5) were plated into 60-mm culture dishes and incubated in the media for 24 h. The media was replaced with serum free DMEM and a mixture containing 5 µg of the plasmid DNA encoding the pleckstrin homology domain of Akt protein kinase (1-167) tagged with green fluorescent protein (PH-Akt-GFP) (24) (kindly provided by Dr. T. Balla, National Institute of Health, Bethesda, MD) and 30 µl of Lipofectamine reagents was gently added to each plate and incubated for 3 h at 37°C at 5% CO_2. The DNA-containing medium was replaced with fresh DMEM containing serum. When co-transfection of Src (wild type) or dominant negative Src (K295R/Y527F) (both were generous gift from Dr. J. Burgge, Harvard medical School, Boston, MA) with PH-Akt-GFP was carried out the DNA concentration used was 1:1. Green fluorescent protein (GFP) plasmid was from Clonetech. SN4741 cells were co-transfected with D_{2L} and PH-Akt-GFP or GFP by calcium phosphate method (25).

Epifluorescence imaging - Microscopy of live cells transfected to express PH-Akt-GFP was performed on the Olympus (B65) upright fluorescent microscope using a water immersion objective lens (x40) fitted with a heated stage and an objective lens heater. Images were collected at 2 min intervals after the addition of the drugs and processed using Adobe Photoshop (5.5).

Immunoblotting and immunoprecipitation- PC12-D_2R cells (1x 10^6 cells/100 mm plate) were grown for 24 and following respective treatments, the cells were washed twice with ice-cold PBS and lysed in buffer 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Igepal C630, 1 mM

phenylmethylsulfonyl fluoride, 1 mM sodium orthovandate, 5 mg/ml aprotinin and cocktail of protease inhibitors (Roche Diagnostics, GmbH) at 4°C for 20 min. After centrifugation at 14,000 x g for 20 min at 4°C, equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis. The resolved proteins were electrotransferred to nitrocellulose membranes. Detection of proteins by immunoblotting was conducted using ECL system according to the manufacturer's recommendations. The blots were then stripped in buffer containing 62.5 mM Tris HCl (pH 6.8), 2% SDS and 100 mM β-mercaptoethanol for 30 min at 50°C and re-probed with respective antibodies

For immunoprecipitation, the protein extract was incubated sequentially (2 h for each incubation at 4°C) with anti-D_2 receptor antibody and protein A/G agarose with gentle agitation. Immunoprecipitates were washed three times with lysis buffer, boiled for 5 min in 3X Laemmli sample buffer, and processed for Western blotting using EGFR antibody. The blots were stripped and reprobed with anti-phospho tyrosine (PY20) antibody.

RNA interference – Custom SMARTpool plus small interfering RNA (siRNA) to target rat EGFR (Cat. No. M-004710-00) was designed and synthesized by Dharmacon (Lafayette, CO). siRNA (50 pmol) was co-transfected with PH-Akt-GFP (2 µg) into PC12-D_2R cells using transit-TKO and -neural transfection reagents (Mirus, Madison, WI) according to manufacturer's protocol. For immunofluorescence, 24 h upon transfection, cells were serum starved for 1h and were treated with bromocriptine for 10 min. The cells were fixed with ice-cold methanol and immunostained for EGFR and visualized using CY 3 conjugated secondary antibody. A non-specific RNA duplex (Dharmacon, Cat. No. D-001206-09-05) was used in control experiments.

RESULTS

Neuroprotection by D_2 receptor activation involves PI 3-kinase/Akt signaling cascade

We have previously reported that the increased cell survival in PC12-D_2R cells mediated by D_2 receptor activation was completely abolished by inhibitors of PI 3-kinase, suggesting that the D_2 receptor may be altering cell survival by activating PI 3-kinase (17). We therefore studied whether PI 3-kinase/Akt signaling was modulated by the D_2 receptor when complexed with an agonist that prevents apoptosis in these cells. Activation of PI 3-kinase generates phosphatidylinositol 3,4,5-triphosphate (PIP3) and thereby stimulates anti-apoptotic proteins (26). The downstream PI 3-kinase target, protein kinase B (Akt), has been reported to be important in mediating survival in many cell types (27). Akt is activated by phosphorylation at Thr^{308} in the catalytic loop and Ser-473 in the C-terminal domain (28,29).

We first determined whether the anti-apoptotic dopamine agonist bromocriptine induced phosphorylation of Ser-473 of endogenous Akt in PC12-D_2R cells. As shown in Fig. 1A, Akt phosphorylation was increased 15 min after exposure to bromocriptine. In some cell lines, H_2O_2 has been reported to activate Akt (30,31). However, we found that in PC12-D_2R cells, H_2O_2 alone had no effect on the phosphorylation of Akt (Fig. 1A). Akt phosphorylation occurs after it is recruited to the plasma membrane through an interaction of its N-terminal pleckstrin homology (PH) domain with PIP3 (32), thereby bringing the enzyme into the proximity of additional PIP3-dependent and -independent protein kinases (33). We studied the redistribution of Akt by D_2 receptor signaling using a PH-Akt-GFP fusion protein (24). The localization of PH-Akt-GFP in quiescent PC12-D_2R cells was indistinguishable from that of transfected GFP alone. Receptor

activation by bromocriptine, however, caused a rapid (<5 min) translocation of the PH-Akt-GFP

to ruffled membrane regions (Fig. 1B, top panels). No response to bromocriptine was observed

in control PC12-D$_2$R cells expressing GFP alone (Fig. 1B, middle panels) or in the parent PC12

cells, which lack the D$_2$ receptor, expressing PH-Akt-GFP (Fig. 1B, bottom panels). We also

tested whether this pathway was active in cells exposed to oxidative stress. As shown in Fig.

1C, the bromocriptine-induced phosphorylation of Akt and translocation of PH-Akt-GFP (Fig.

1D) were unaffected in the presence of H$_2$O$_2$. Thus, D$_2$ receptor stimulation by bromocriptine

caused translocation and phosphorylation of Akt in PC12-D$_2$R cells during oxidative stress.

D$_2$ receptor activation of Akt is PTX insensitive and PI 3-kinase dependent

The D$_2$ receptor is a member of the rhodopsin-like heptahelical receptor family, whose

classical signaling pathway involves the activation of the Gi/Go subtype heterotrimeric G-

proteins, which can be inactivated by PTX (34). In order to examine the role of Gi/Go coupling

in activation of PI 3-kinase/Akt, we determined the effects of PTX on these responses.

Pretreatment of PC12-D$_2$R cells with 100 ng/ml PTX (16 h) eliminated bromocriptine-stimulated

[^{35}S]GTPγS binding (data not shown). In contrast, the D$_2$ receptor-mediated phosphorylation of

Akt was unaffected by PTX (Fig. 2A, compare lane 3 and 4). To assess the role of PI 3-kinase in

the activation of Akt induced by D$_2$ receptors, PC12-D$_2$R cells were pretreated with the inhibitor

wortmannin at 100 nM, a concentration that selectively blocks PI 3-kinase (35). The cultures

were then exposed to bromocriptine. Wortmannin completely prevented the phosphorylation of

Akt that is inducible by D$_2$ receptor activation (Fig. 2A, compare lanes 4 and 5). Similar results

were also obtained with LY294002 (20 μM) another commonly used but less potent inhibitor of

PI 3-kinase (35) (Fig. 2A, compare lane 4 and 6). Translocation of PH-Akt-GFP by

bromocriptine was also inhibited by pretreatment with the PI 3-kinase inhibitor wortmannin, while it was unaffected by PTX pretreatment (Fig. 2B). Similar results were obtained with LY294002 (data not shown). These results indicate that the D_2R-mediated activation of Akt occurs through PI 3-kinase by a mechanism independent of Gi/Go class heterotrimeric G-proteins.

D_2 receptor activates PI 3-kinase/Akt pathway in nigral dopamine cells

To explore whether the D_2 receptor coupling to PI 3-kinase/Akt observed in PC12-D_2R cells was present when the D_2 receptor was expressed in a different cellular context, we studied this signaling pathway in the mouse immortalized nigral dopamine cell line SN4741, which expresses tyrosine hydroxylase, the dopamine transporter and D_2 autoreceptors (23). Activation of D_2 receptors by bromocriptine in these cells was found to induce phosphorylation of endogenous Akt (Fig. 3A). When the cells were transfected with the PH-Akt-GFP construct, bromocriptine induced redistribution of this reporter (data not shown). The capacity of the activated D_2 receptor to induce Akt redistribution in this model was enhanced in cells co-transfected with D_2 receptor and PH-Akt-GFP. Bromocriptine caused a translocation of the PH-Akt-GFP protein into discrete regions of the SN4741 cells (Fig. 3B) that was similar to the response observed in differentiated PC12-D_2R cells (Fig. 3C). The bromocriptine-stimulated translocation of PH-Akt-GFP in SN4741 cells was eliminated by pre-treatment with the PI 3-kinase inhibitor LY290042 (data not shown). Control experiments in which cells were transfected with the D_2 receptor and GFP showed no change in the distribution of fluorescence in response to bromocriptine. These results suggest that the D_2 receptor can couple to the PI 3-kinase/Akt signaling pathway in dopaminergic neurons.

D$_2$ receptor-mediated neuroprotection and activation of PI3-kinase/Akt involves EGFR transactivation

We previously reported that bromocriptine showed significant PI 3-kinase-dependent anti-apoptotic activity in PC12-D$_2$R cells and have demonstrated, as described above, that bromocriptine also induced Akt phosphorylation and translocation. We next attempted to delineate the signal mediators connecting the D$_2$ receptor to PI 3-kinase. It has been reported that the PI 3-kinase/Akt pathway in PC12 cells can be activated by receptor tyrosine kinases (36). The effectiveness of several receptor tyrosine kinase inhibitors on bromocriptine-mediated neuroprotection was evaluated. H$_2$O$_2$ exposure caused significant loss of PC12-D$_2$R cell viability at 24 h, as determined using the MTT metabolism assay, and this cell loss was nearly completely reversed by the D$_2$ receptor agonist bromocriptine (Fig. 4A), consistent with our previous results (17). The effects of various growth factor receptor inhibitors on the capacity of bromocriptine to protect cells against cell death due to H$_2$O$_2$ exposure were studied. AG1296 (200 nM), AG1478 (200 nM), and k252a (50 nM) in the presence of H$_2$O$_2$ and the presence or absence of bromocriptine (100 nM) for 24 h were evaluated. As shown in Fig. 4A, AG1478, a specific inhibitor of EGFR intrinsic tyrosine kinase activity (37), completely abolished the neuroprotection provided by bromocriptine exposure, an effect similar to that observed with inhibition of PI 3-kinase (17). In contrast, inhibition of platelet-derived growth factor (PDGF) receptors by AG1296 or NGF receptor by k252a had no effect on D$_2$ receptor mediated cell survival.

We next investigated the role of the EGFR in mediating the signaling from the D$_2$ receptor to Akt. Activation of the EGFR by EGF caused a rapid phosphorylation of Akt and a

translocation of PH-Akt-GFP in PC12-D$_2$R cells, similar to the response observed with bromocriptine (Fig. 4B). The involvement of EGFR transactivation in D$_2$ receptor-stimulation of Akt phosphorylation and translocation was supported by finding a complete inhibition of these responses after pretreatment with AG1478 (Fig. 4C). These results suggest that PI 3-kinase/Akt is one of the downstream effectors of the EGFR and that the D$_2$ receptor activates PI 3-kinase/Akt via transactivation of the EGFR in PC12-D$_2$R cells.

To confirm the role of the EGFR in the activation of PI-3 kinase/Akt by D$_2$ receptors, we reduced the levels of EGFR expression in PC12-D$_2$R cells using RNA interference. After transfection with EGFR-specific or control small interfering RNA (siRNA), cultures were assessed for EGFR protein expression by immunofluorescence. As shown in Fig. 5, EGFR was substantially repressed by 24 h post transfection in approximately 60-70% of the cells. The involvement of EGFR transactivation in D$_2$ receptor-stimulation of Akt was studied in PC12-D$_2$R cells co-transfected with PH-Akt-GFP and EGFR siRNA or control siRNA. Following 24 h post transfection the cells were serum starved for 1 h, stimulated with bromocriptine and assessed for the expression of EGFR and the translocation of PH-Akt-GFP we demonstrated above. In control siRNA transfected cells, PH-Akt-GFP translocation was similar to that observed in cells not tranfected with siRNA (Fig. 6A and data not shown). However, EGFR repression by siRNA completely blocked the translocation of PH-Akt-GFP by bromocriptine (Fig. 6B). We conclude that EGFR is essential for the translocation of PH-Akt-GFP following D$_2$ receptor stimulation.

In order to clarify the mechanisms through which the D$_2$ receptor transactivates the EGFR, we examined the association between these two membrane proteins. We performed

immunoprecipitations using an anti-D_2 receptor antibody in the presence and absence of bromocriptine and immunoblotted for the EGFR (Fig. 7). These studies showed that the EGFR co-immunoprecipitated with the D_2 receptor and the association between these two proteins was augmented in the presence of bromocriptine. Furthermore, the EGFR that complexed with the D_2 receptor in the presence of bromocriptine showed an increase in Tyr-phosphorylation. These data indicate that the D_2 receptor and EGFR form a complex and that their association is augmented by bromocriptine.

The sites of EGFR Tyr phosphorylation induced by bromocriptine were studied using site-specific anti-phosphotyrosine antibodies. We analyzed tyrosine phosphorylation of the EGFR at residues 992 and 1068, which are EGFR autophosphorylation sites (38) and at residue 845 (Tyr-845), a known Src phosphorylation site (39). As shown in Fig. 8, incubation of PC12-D_2R cells with EGF increased the phosphorylation of tyrosine residues 845, 992 and 1068, whereas, bromocriptine only enhanced phosphorylation of Tyr-845. The bromocriptine-mediated phosphorylation of Tyr-845 was inhibited by pretreatment with EGFR inhibitor (Fig. 8C).

EGFR transactivation is c-Src dependent

The finding that bromocriptine enhanced phosphorylation of Tyr-845, a Src-dependent phosphorylation site of the EGFR (39), led us to study the role of c-Src in this signaling. Src family kinases have been implicated in the phosphorylation of the EGFR and of PI 3-kinase (39-41). We examined the phosphorylation of Tyr-418 in c-Src, which is an autophosphorylation site required for kinase activity of c-Src (42). Cells were exposed to bromocriptine (100 nM) for periods up to 30 min. To determine whether c-Src was activated by D_2 receptor stimulation, we

performed immunoblotting using an antibody specific for c-Src phospho-Tyr-418. Stimulation of the D_2 receptor by bromocriptine caused c-Src to be phosphorylated at Tyr-418 (Fig. 9A).

We then examined the effects of the Src family tyrosine kinase inhibitor PP2 on D_2 receptor mediated Akt phosphorylation and cell survival. PP2 completely inhibited both the capacity of bromocriptine to induce phosphorylation of Akt (Fig. 9B) and to mediate cell survival in the presence of oxidative stress (data not shown). PP2 also prevented the ability of bromocriptine treatment to induce the phosphorylation of c-Src-Tyr-418 and EGFR-Tyr-845 (Fig. 8C). However, inhibition of the EGFR by AG1478 did not affect the capacity of bromocriptine to induce phosphorylation of c-Src (Fig. 9C), suggesting that the EGFR is downstream of c-Src in D_2 receptor signaling. The role of c-Src in the D_2 receptor signaling was further evaluated using a dominant negative c-Src construct. When co-expressed with PH-Akt-GFP, the dominant negative c-Src kinase (k295R/Y527F) completely abolished translocation of PH-Akt-GFP in response to bromocriptine (Fig. 9D). Thus both pharmacological inhibition and dominant negative studies indicate that c-Src activation is required for signaling from the D_2 receptor through the EGFR to the neuroprotective PI 3-kinase/Akt pathway.

Translocation and phosphorylation of Akt by D_2 receptor stimulation are agonist-specific

We had previously found that D_2 receptor agonists varied greatly in their capacity to mediate increased survival of PC12-D_2R cells and that their protective efficacy showed no correlation with G-protein activation, as assayed by GTPγS binding. In particular, the efficacy of the agonists bromocriptine and pramipexole for GTPγS-binding were indistinguishable, whereas pramipexole was essentially devoid of neuroprotective activity in the PC12-D_2R model. Having implicated the PI 3-kinase/Akt signaling pathway in the neuroprotection mediated by the

D_2 receptor when complexed with bromocriptine, we were interested in determining the effects of pramipexole on this pathway. As shown in Fig. 10, pramipexole failed both to induce translocation of PH-Akt-GFP and phosphorylation of Akt in PC12-D_2R cells. These results suggest that specific agonists that interact with the dopamine D_2 receptor differ markedly in their relative activation of classical and growth factor signaling pathways when complexed with the D_2 receptor (see discussion).

DISCUSSION

We have delineated a D_2 receptor-activated signaling pathway that mediates neuroprotection by specific D_2 agonists in dopaminergic cell lines. Bromocriptine stimulates the PI 3-kinase/Akt pathway through a PTX-insensitive mechanism involving c-Src and transactivation of the EGFR. Our results suggest that the relative activation of classical G-protein and growth factor signaling pathways by the D_2 receptor is agonist specific.

Since bromocriptine can induce the activation of the PI 3-kinase/Akt pathway and in many circumstances the modulation of Akt signaling normally occurs via growth factor stimulation, we sought to determine if the effects of bromocriptine on PC12-D_2R cells were mediated through a growth factor receptor. Here, we report that the bromocriptine induced the activation of Akt within minutes and this activation required the EGFR. We show that EGFR specific tyrosine kinase inhibitor completely block bromocriptine induced activation of Akt. Furthermore, EGFR repression by siRNA also inhibited the translocation of PH-Akt-GFP by bromocriptine. Inhibitor and dominant negative Src studies show that the activation of the EGFR by the D_2 receptor involves Src. Co-immunoprecipition studies show that the D_2 receptor complexes with the EGFR and that this association is enhanced by D_2 receptor activation. The neuroprotective D_2 receptor signaling pathway we have characterized is summarized in Fig. 11.

Our data indicate that stimulation of c-Src /EGFR family kinases are required for Akt activation in response to bromocriptine in PC12-D_2R cells. Src family kinases have been implicated in GPCR-induced EGFR tyrosine phosphorylation, and GPCRs can induce association of Src with the EGFR (43-45). In other studies, GPCR-induced EGFR tyrosine

phosphorylation was found to be Src independent (46,47). We found that bromocriptine caused activation of the EGFR and inhibition of Src kinases had significant effect on bromocriptine-induced EGFR tyrosine phosphorylation, implicating Src family kinases in bromocriptine-induced EGFR tyrosine phosphorylation. Src kinases can be activated by several heptahelical receptors (43,48,49) as well as by growth factor receptor stimulation (50), including the EGFRs (39,43,51). Src has been reported to influence EGFR activity by mediating phosphorylation of Tyr-845, a consensus c-Src phosphorylation site in the EGFR (39). Inhibition of either c-Src or the EGFR impaired the ability of bromocriptine to cause activation of Akt in PC12-D_2R cells, indicating that activation of both proteins are required for this signaling. Inhibition of Src kinase also inhibited EGFR phosphorylation at Tyr-845, whereas inhibition of the EGFR did not prevent phosphorylation of c-Src. These results suggest that c-Src is upstream of both the EGFR and Akt. Moreover, inhibiting either c-Src or the EGFR completely abolished the capacity of bromocriptine to increase cell survival during oxidative stress. Although Akt has been reported to be a substrate for c-Src phosphorylation (52,53), in PC12-D_2R cells both c-Src and EGFR phosphorylation were required for Akt activation. Therefore we propose that in dopaminergic neurons, the D_2 receptor transactivates the EGFR through c-Src, which in turn activates the cytoprotective PI 3-kinase/Akt pathway.

Several mechanisms have been reported for heptahelical receptor activation of Src kinase. The β3 adrenergic receptor interacts with c-Src directly via Pro rich domains in the receptor (49). Src activation by the β2 adrenergic receptor requires arrestin (48). The D_3 receptor has been found to contain non-canonical SH3 ligands (54). Putative SH3 domains are also present in the D_2 receptor, which might potentially mediate an interaction with Src. Using co-

immunoprecipitation studies, we have demonstrated that the D_2 receptor and the EGFR form a complex. Our inhibitor and dominant negative Src data suggest that c-Src might form a component of this complex. Unfortunately, this hypothesis could not be tested directly in co-immunoprecipitation studies due to the relatively poor sensitivity of the anti-Src antisera. Whether the activation of c-Src by the D_2 receptor occurs directly or requires additional adaptor proteins remains to be determined.

Heptahelical receptors, including D_2-class receptors, have been reported to induce activation of growth factor receptor-coupled pathways or PI 3-kinase (49,55-64). The cellular background in which a receptor is expressed may be important in determining its signaling potential. Platelet-derived growth factor receptor (PDGF) transactivation by the D_2 and D_4 receptors expressed in CHO cells has been reported (60). However, in contrast to our results for EGFR phosphorylation, the transactivation of the PDGF receptor in CHO cells showed sensitivity to PTX (60). The D_3 receptor expressed in CHO cells mediates activation of PI 3-kinase via atypical protein kinase C in a manner also sensitive to PTX (63). In striatal neurons, D_2 agonist has been reported to activate Akt independently of PI3-kinase activation (65). However, we find in both PC12-D_2R and the dopaminergic SN4741 cells that Akt activation requires PI 3-kinase activity. Our results in the two dopamine cell lines studied are consistent with the observations of Kihara et al. in cortical neurons, who also found that bromocriptine activated the PI 3-kinase/Akt pathway (62).

Our investigations further suggest that the D_2 receptor, when activated by bromocriptine, can couple both to heterotrimeric Gi/Go family G-proteins and, simultaneously, to the PI3-kinase/Akt signaling pathway (Fig. 11). The data lead us to propose that the coupling to

heterotrimeric G-protein and the coupling to PI 3-kinase/Akt may be independent. Firstly, the

G-protein coupling, but not the Akt activation showed PTX sensitivity. Secondly, the agonists

studied differed in their capacity to activate each pathway. Bromocriptine activated both

signaling pathways, whereas pramipexole, while quite efficient at simulating GTPγS binding in

these cells (17), failed to activate the PI 3-kinase/Akt signaling pathway (See Fig. 10). Our data

suggest that the anti-apoptotic activity induced by dopamine agonists in these cells resulted from

transactivation of the PI 3-kinase/Akt pathway. In a previous study, we found little correlation

between the capacity of agonists to confer protection against oxidative stress and their capacity

to activate classical G-protein signaling (17). Based on these results, we propose a model where

the specificity of the agonist complexed with the D_2-receptor determines the switching of

signaling between G-protein and growth factor signaling pathways.

Kenakin originally proposed that specific agonists acting at the same receptor might

differentially activate downstream signaling pathways, a phenomenon he called agonist-mediated

signal trafficking (13). Signal trafficking could arise as a result of receptors having multiple active

conformational states that differ in their activation of specific signaling pathways. Agonists

could cause different patterns of signaling by each inducing a different relative distribution of the

accessible active states. Many studies suggest that heptahelical receptors exhibit properties

consistent with the existence of multiple conformational states. In rhodopsin, for example, the

existence of multiple conformers is evident from absorbance changes (9). Multiple receptor

conformational states are also evident in single molecule spectroscopy studies of the β2-

adrenergic receptor (11) and are supported by the presence of phenotypically different serotonin

5HT2C receptor activation mutants (12). Pharmacological evidence for signal trafficking has been

reported in several heptahelical receptors (14,15,66,67). Evidence for signal trafficking at the D_2 receptor based on the G-protein sensitivity of binding affinity has been previously reported for D_2 receptor expressing Sf21 insect cell lines. Notably, the agonist bromocriptine was found to induce a distinct pattern of coupling (15). Our results are consistent with the signal trafficking hypothesis and suggest that agonists acting at the D_2 receptor may differ markedly in their capacity to stabilize conformations leading to classical and growth factor signaling.

We implicate the capacity of D_2 agonists in transactivating the PI3-kinase/Akt pathway and in mediating anti-apoptosis in PC12-D_2R cells. Furthermore, we find evidence that the effectiveness of an agonist to protect against oxidative stress by activating PI 3-kinase/Akt may differ greatly for specific agonists. Our results suggest the hypothesis that agonists have a conformationally specific effect at the D_2 receptor. Among the agonists studied to date, we find agonists that preferentially activate GTPγS binding and agonists that activate both GTPγS binding and anti-apoptotic signaling. Our results suggest that it may be possible to identify agonists that specifically traffic signaling to the EGFR-PI 3-kinase/Akt pathway in dopamine neurons. Given the central role of the dopamine D_2 receptor in brain function, the refined model of conformationally dependent D_2 receptor signaling has important implications for the pathophysiology and treatment of brain diseases involving altered dopamine neuronal survival or neurotransmission, such as Parkinson's disease and Schizophrenia.

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Figure Legends

Figure 1. D_2 receptor stimulation activates Akt in PC12- D_2R cells. (A) Phosphorylation of

Akt by D_2 receptor agonist bromocriptine. PC12- D_2R cells were stimulated with 1 μM of

bromocriptine or H_2O_2 (200 μM) for the periods of time indicated. After stimulation, cells

lysates were prepared and analyzed by western blotting with antiphospho-Akt antibody or with

anti-Akt antibody. (B) Translocation of PH-Akt-GFP by D_2 receptor stimulation. PC12- D_2R

cells expressing PH-Akt-GFP (48 h after transfection) were stimulated with D_2 agonist

bromocriptine (100 nM) and translocation of PH-Akt-GFP was determined by live cell imaging.

Each pair of left (0 min) and right (10 min) frame represents the images captured from the same

living cell. Bromocriptine (100 nM) stimulates translocation of PH-Akt-GFP reporter to the

membrane within 10 min (top frames). Arrows indicate localized areas of PH-Akt-GFP

translocation following addition of bromocriptine. Bromocriptine (100 nM) had no effect on the

translocation of or EGFP expressed in PC12- D_2R cells (middle frames) or PH-Akt-GFP

expressed in native PC12 cells, which lack D_2 receptors (bottom frames). Frames shown are from

1 of 8 independent experiments. (C) Oxidative stress had no effect on the phosphorylation or

translocation of Akt in presence of bromocriptine. Western blot of phospho-Akt. Lane 1,

control; lane 2, bromocriptine (1 μM); lane 3, bromocriptine plus H_2O_2 (200 μM). All the

incubations were carried out for 15 min. (D) Bromocriptine induced translocation of PH-Akt-

GFP was unaffected in presence of H_2O_2 (200 μM). Live cell imaging was carried out following

addition of H_2O_2 (200 μM) and bromocriptine (100 nM) into the medium. Each pair of left (0

min) and right (10 min) frame represents the images captured from the same living cell. Arrows

indicate localized areas of PH-Akt-GFP translocation following addition of bromocriptine. Frames shown are from 1 of 6 independent experiments.

Figure 2. Activation of PI 3-kinase/Akt signaling cascade by D_2 receptor is independent of PTX sensitive G-protein coupled pathway. (A) Western blot showing the phosphorylation of Akt by bromocriptine in presence of PTX, but no stimulation of Akt phosphorylation in presence of PI 3-kinase inhibitors LY 294002 or wortmannin. PC12- D_2R cells were either left untreated or pre-treated with PTX (100 ng/ml; 16h), wortmannin (100 nM) or LY294002 (10 μM) for 1h and then stimulated with 1 μM of bromocriptine for 15 min. (B) Effect of PTX or PI 3-kinase inhibitors on translocation of PH-Akt-GFP by bromocriptine (100 nM). PTX (100 ng/ml, 16h) pretreatment had no effect on the bromocriptine-induced redistribution of PH-Akt-GFP (top frames). Pretreatment of cells with PI 3-K inhibitor wortmannin (100 nM, 1h) completely inhibited the bromocriptine-induced redistribution of PH-Akt-GFP (bottom frames). Arrows indicate localized areas of PH-Akt-GFP following addition of bromocriptine. Frames shown are from 1 of 6 independent experiments.

Figure 3. D_2 receptor activates Akt in immortalized nigral DA cell line (SN 4741). (A) Phosphorylation of endogenous Akt by bromocriptine in SN DA cells (top) and total Akt (bottom). Lane 1, control, lanes 2 and 3, cells incubated (15 min) with 100 nM and 1 μM bromocriptine, respectively. (B) SN4741 cells co-expressing human D_2 receptor and PH-Akt-GFP was stimulated with bromocriptine (100 nM) and live cell imaging was carried out described in Experimental procedures. Bromocriptine stimulated the redistribution of PH-Akt-GFP to discrete areas as indicated by arrows in the cells within 10 min (bottom frame). Frames shown are

from 1 of 6 independent experiments. (C) In differentiated PC12- D_2R cells, bromocriptine (100 nM) stimulated redistribution of PH-Akt-GFP to discrete locations in cell processes as indicated by arrows. Frames shown are from 1 of 6 independent experiments.

Figure 4. (A) Elimination of dopamine agonist mediated neuroprotection by inhibition of EGFR. The EGFR inhibitor AG1478 (200 nM) eliminates the protective action of bromocriptine (100 nM) on H_2O_2-induced apoptosis. Data is plotted from one experiment (mean±SEM, N = 8), representative of four independent experiments. *p<0.001 compared with control (no treatment). #p<0.001 compared with H_2O_2. #*p<0.001 compared with H_2O_2 plus bromocriptine. Inhibition of PDGF receptor by AG1296 (200 nM) or NGF receptors by k252a (50 nM) had no effect on the protection mediated by bromocriptine. (B) Effect of EGFR inhibitor on D_2 receptor-mediated phosphorylation of Akt in PC12- D_2R cells. Cells were either left untreated or pre-treated with EGFR inhibitor, AG1478 (200 nM) for 30 min and then stimulated with 1 µM of bromocriptine for 15 min or EGF (100 ng/ml) for 10 min. After stimulation, cells were lysed and lysates were analyzed by Western blotting with either antiphosphorylated Akt or anti- Akt antibody. (C) Effect of EGFR inhibitor on D_2 receptor-mediated translocation of Akt in PC12- D_2R cells. EGFR inhibition impaired PH-Akt-GFP translocation following D_2 receptor stimulation. EGF (100 ng/ml) translocates PH-Akt-GFP to discrete areas (top panels) and pretreatment with AG1478 (200 nM, 30 min) inhibit PH-Akt-GFP translocation by bromocriptine (bottom panels).

Figure 5. Gene silencing of EGFR by RNA intereference. PC12-D_2R cells were tranfected with either control siRNA or EGFR siRNA as described in the Experimental Procedures section. The

cells were immunostained with anti-EGFR antibody 24 h after transfection. The EGFR

expression was assessed by immunofluorescence microscopy. Note the substantial loss of EGFR

immunoreactivity in the majority of EGFR-siRNA transfected cells.

Figure 6. Gene silencing of EGFR inhibits D_2 receptor signaling to Akt. EGFR siRNA inhibited

bromocriptine-induced PH-Akt-GFP translocation. PC12-D_2R cells were transfected with EGFR

siRNA or control siRNA together with PH-Akt-GFP plasmid DNA. The cells were treated with

bromocriptine (100 nM) for 10 min and PH-Akt-GFP translocation and EGFR expression were

examined in these cells. Each set of three vertical panels represents the same field. EGFR

immunofluorescence is indicated in red (top panels). PH-Akt-GFP signal is indicated in green

(middle panels). The bottom panels are overlays of both EGFR and PH-Akt-GFP signals. (A)

Bromocriptine induced a characteristic ring-like margination of PH-Akt-GFP and concentration

of PH-Akt-GFP into membrane processes in cells transfected with control siRNA. (B)

Suppression of EGFR expression by EGFR siRNA eliminated the redistribution of PH-Akt-GFP

by bromocriptine. Data shown are representative of three independent experiments.

Figure 7. Bromocriptine induced the association of D_2 receptor with EGFR. PC12-D_2R cells

were treated with either vehicle or 1 μM bromocriptine for 10 min. After cell lysis, D_2 receptor

was immunoprecipitated with a monoclonal anti D_2 receptor antibody. Following

immunoblotting, the EGFR was detected using anti-EGFR antibody (upper panel). Stripping

and reprobing with monoclonal anti-phosphotyrosine antibody revealed EGFR tyrosine

phosphorylation associated with bromocriptine stimulated D_2 receptor (lower panel). The blot

shown is representative of three independent experiments.

Figure 8. Tyrosine phosphorylation of residue 845 of the EGFR by bromocriptine. PC12- D_2R

cells were incubated with 1 μM bromocriptine for the indicated time points and EGF for 10 min. The cells were then lysed, and the lysates were resolved by SDS-gel electrophoresis on 7.5% gel and immunoblotted. Tyrosine phosphorylation was detected using site-specific tyrosine antibodies to EGFR from total cell lysates using the indicated anti-phosphotyrosine antibodies. EGFR levels were detected using anti-EGFR antibody. (A) Bromocriptine had no effect on the phosphorylation of EGFR Tyr0-992 and Tyr-1068. (B) Bromocriptine induced phosphorylation of EGFR Tyr-845. (C) Phosphorylation of EGFR Tyr-845 by bromocriptine was inhibited by AG1478 pre-treatment (200 nM, 30 min).

Figure 9. Bromocriptine stimulates the activation of the c-Src tyrosine kinase. (A) PC12- D_2R cells were incubated with 1 μM of bromocriptine for the indicated time points and EGF for 10 min. Tyrosine phosphorylation of site-specific tyrosine residues of c-Src was detected from total cell lysates using the anti-phospho Tyr-418 antibody. Total c-Src level was detected using anti-Src antibody. (B) PP2 treatment inhibits bromocriptine-induced phosphorylation of Akt. PC12- D_2R cells were either left untreated or pretreated with 1 μM PP2, AG1478 (200 nM) or wortmannin (100 nM) for 30 min. The cells were then stimulated with 1 μM bromocriptine for 15 min. The cells were then lysed, and the lysates were resolved by SDS-gel electrophoresis on a 12% gel and immunoblotted with either anti-phosphotyrosine 418 antibody or anti-Src antibody. (C) Inhibition of Src-kinase abolished bromocriptine-mediated phosphorylation of c-Src and EGFR. (D) Inhibition of c-Src kinase activity prevents redistribution of PH-Akt-GFP in response to stimulation of the D_2 receptor by bromocriptine. PC12- D_2R cells were co-transfected with wild type Src or dominant negative Src (K295R/Y527F) and analyzed by epifluorescence microscopy. Redistribution of PH-Akt-GFP occurs in the presence of wild-type
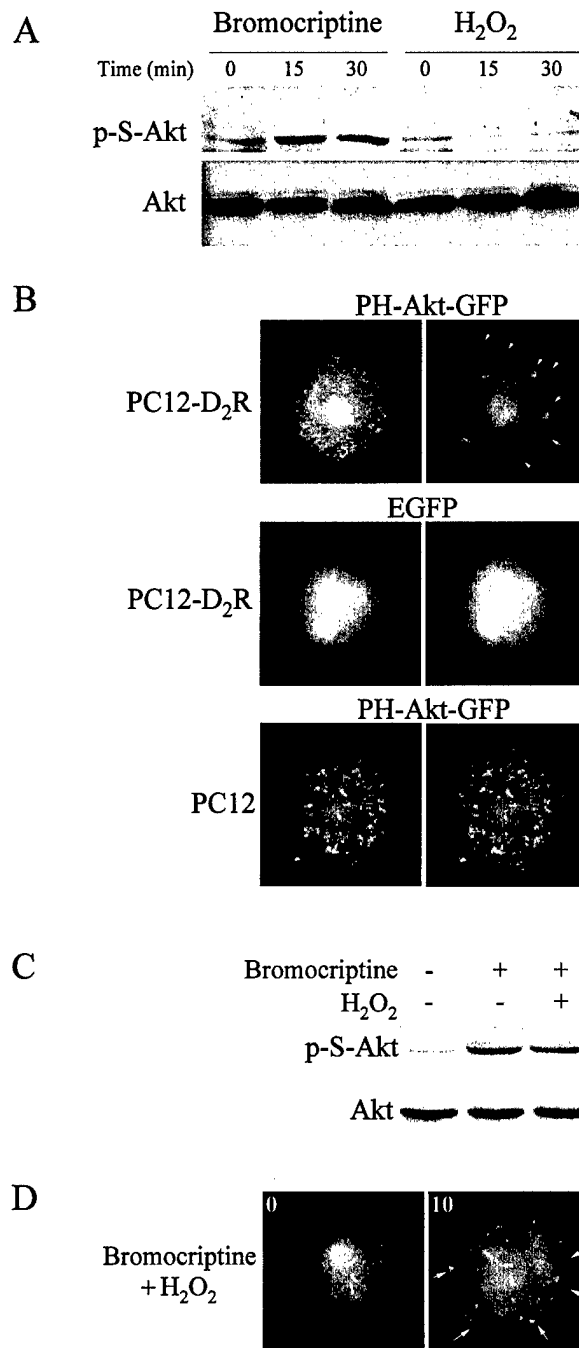
c-Src (top panels, see arrows) but not in the presence of dominant negative c-Src (bottom panels).

Figure 10. Differential phosphorylation of endogenous Akt by D_2 receptor agonists. (A) Western blot showing the differential phosphorylation of Akt by bromocriptine and pramipexole. Lane 1, control; lane 2, bromocriptine; and lane 3, pramipexole. The concentration of bromocriptine and pramipexole used was 1 μM for 30 min incubation. (B) Graphical representation of Akt phoshorylation in response to bromocriptine and pramipexole. Bromocriptine (1 μM) significantly increased (* $p<0.05$) Akt phosphorylation within 15 min after the addition of the drug, whereas, pramipexole (1 μM) showed no effect on the phosphorylation of Akt. Data are mean$\pm$ SEM values from one experiment performed in triplicate, representative of three independent experiments. (C and D) In contrast with bromocriptine, pramipexole had no effect on the translocation of PH-Akt-GFP in PC12-D_2R cells.

Figure 11. Schematic of signaling pathways modulated by the D_2 receptor. Dopamine D_2 receptor activates PI 3-kinase/Akt pathway via c-Src and the EGFR. The D_2 agonist bromocriptine, but not pramipexole, activates the c-Src/EGFreceptor/PI 3-kinase/Akt pathway.
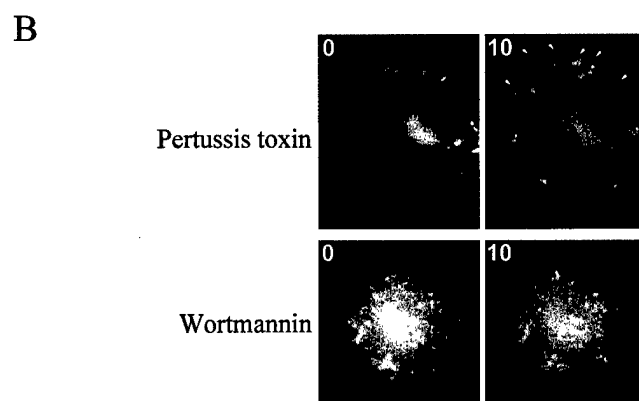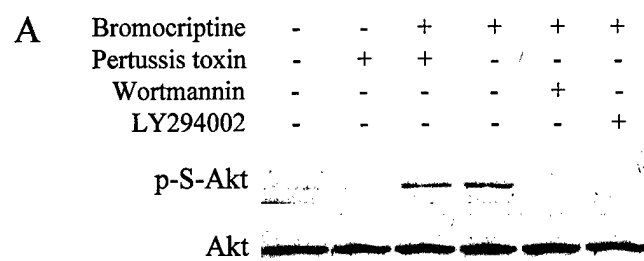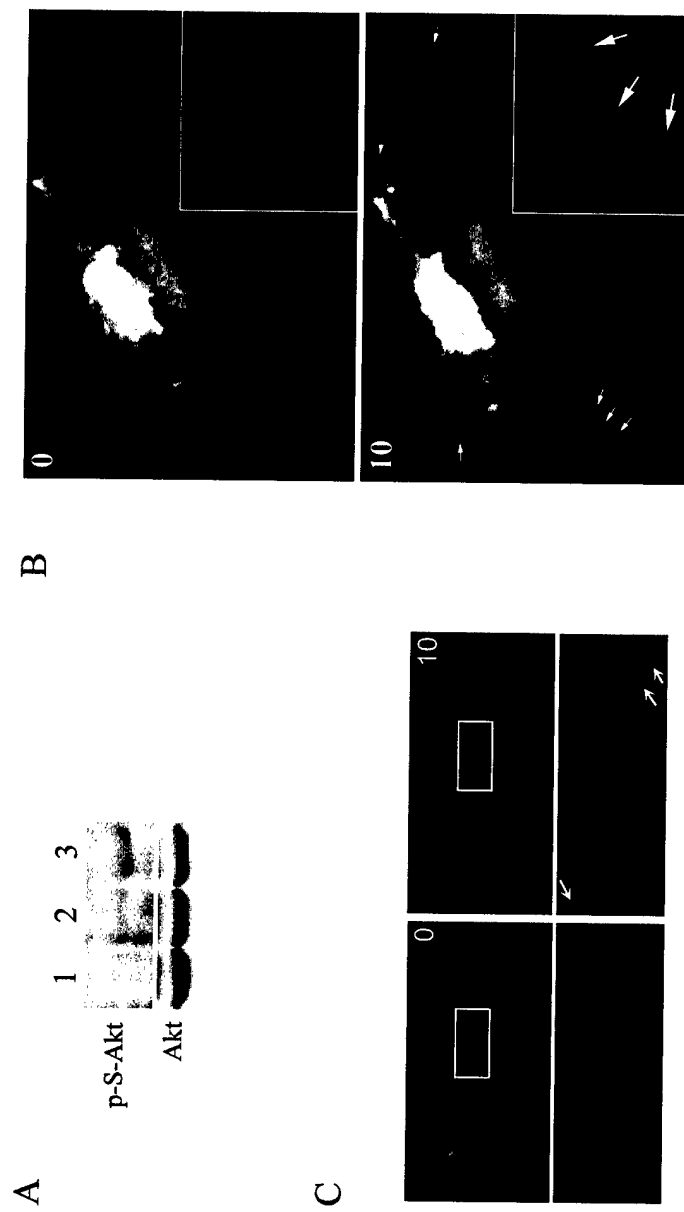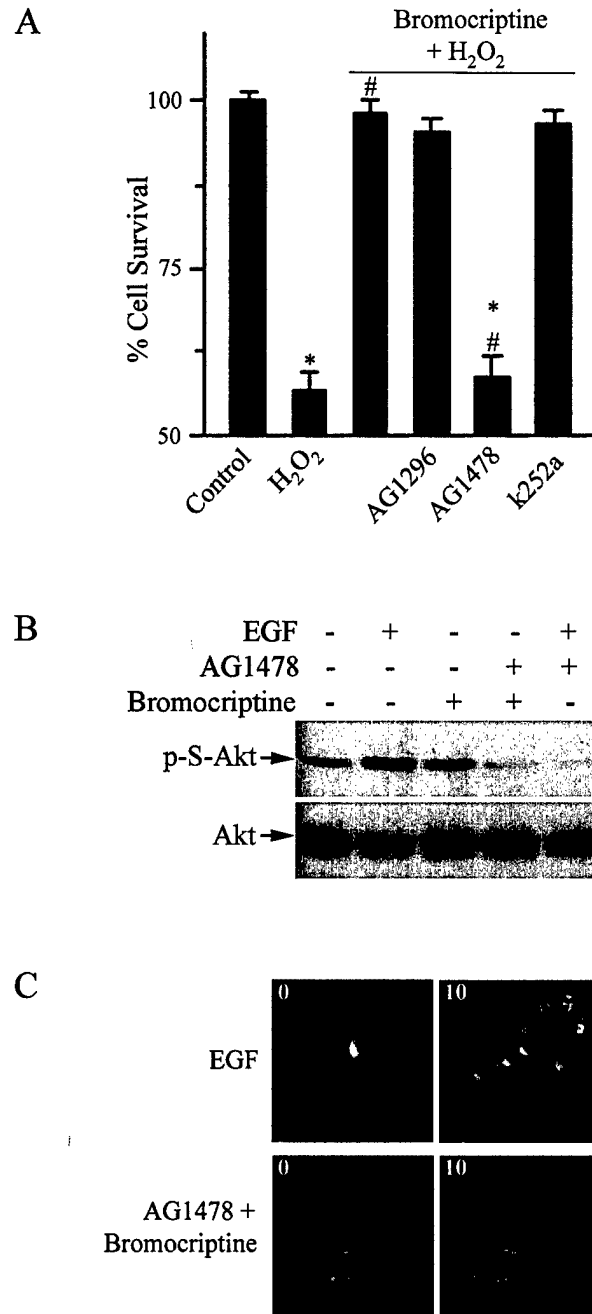
Figure 1

A

	Bromocriptine			H_2O_2		
Time (min)	0	15	30	0	15	30

p-S-Akt

Akt

B

PH-Akt-GFP

PC12-D$_2$R

EGFP

PC12-D$_2$R

PH-Akt-GFP

PC12

C

Bromocriptine	-	+	+
H_2O_2	-	-	+

p-S-Akt

Akt

D

Bromocriptine +H_2O_2

Figure 2

A

Bromocriptine	-	-	+	+	+	+
Pertussis toxin	-	+	+	-	-	-
Wortmannin	-	-	-	-	+	-
LY294002	-	-	-	-	-	+

p-S-Akt

Akt

B

Figure 3

A

p-S-Akt

Akt

B

C

Figure 4

A

B

C

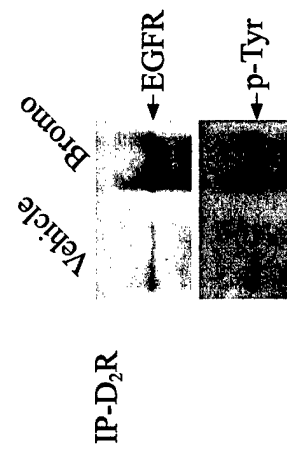**Figure 5**

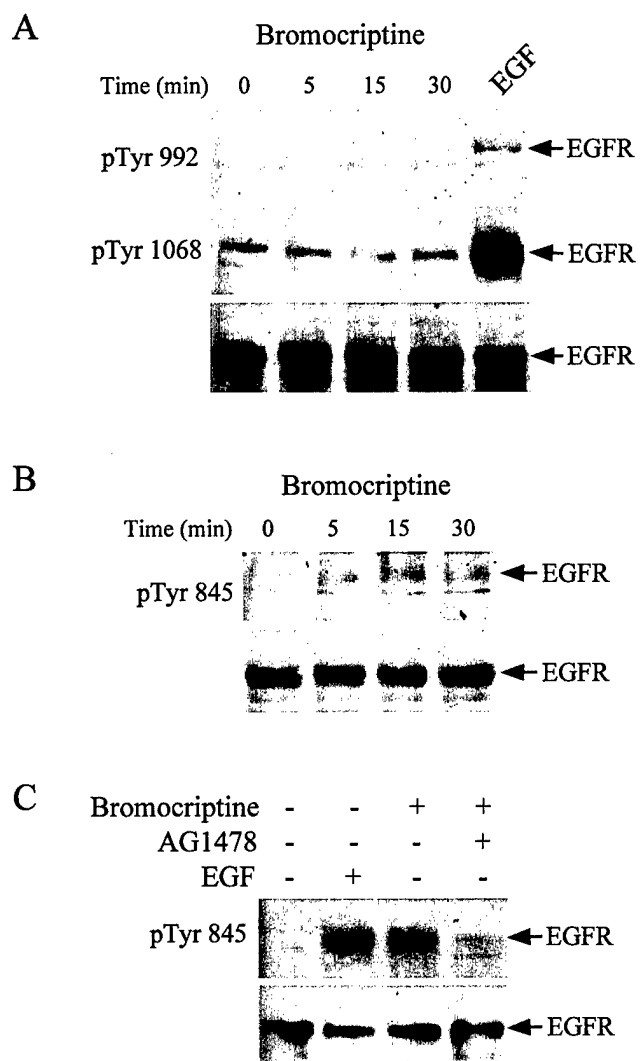Control-siRNA   EGFR-siRNA
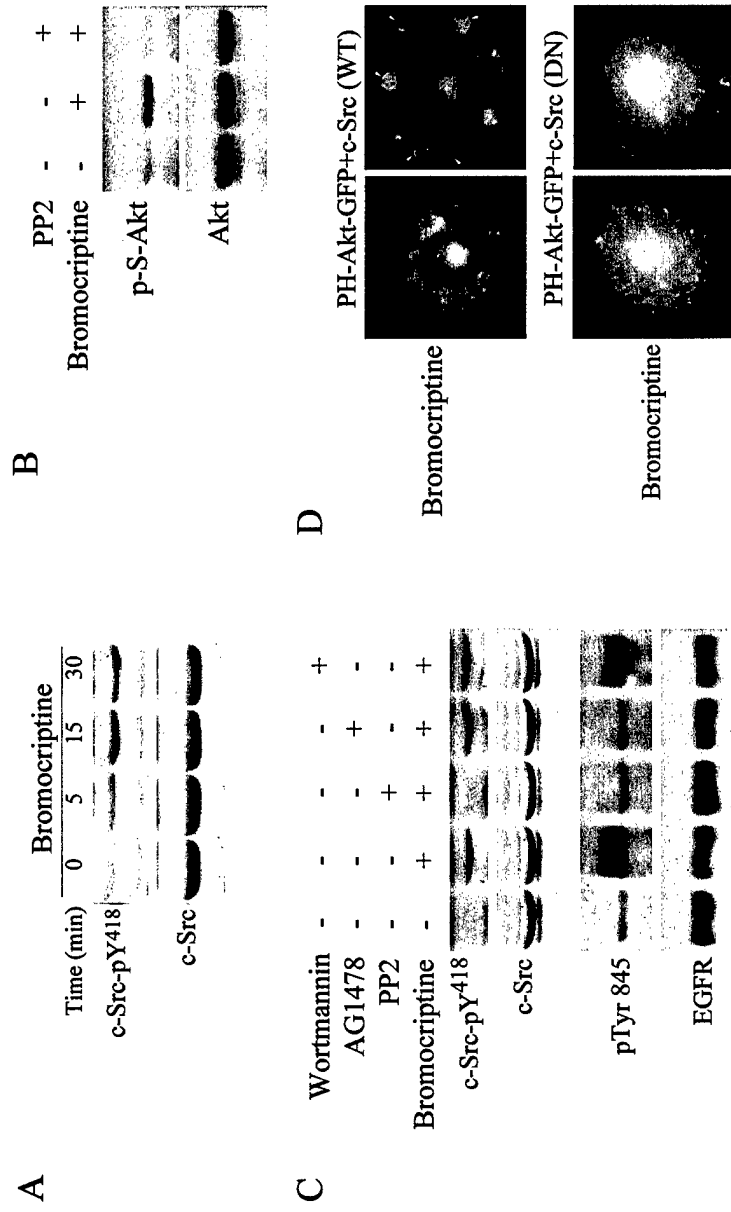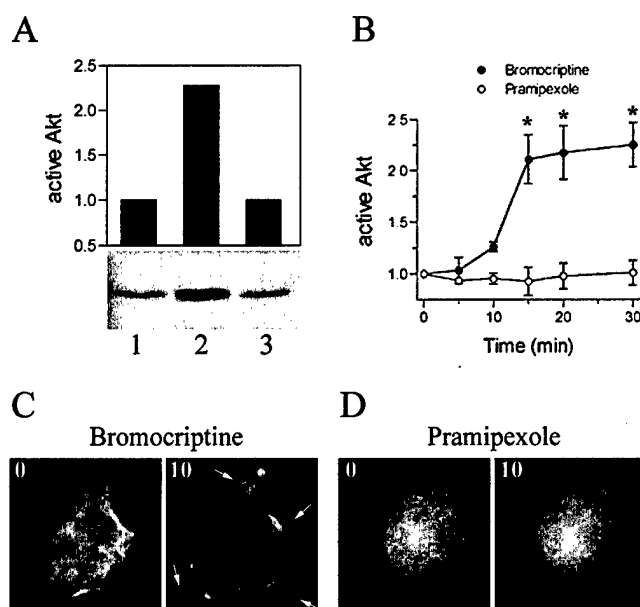
Figure 6

Figure 7

IP-D₂R

Figure 8

A

B

C
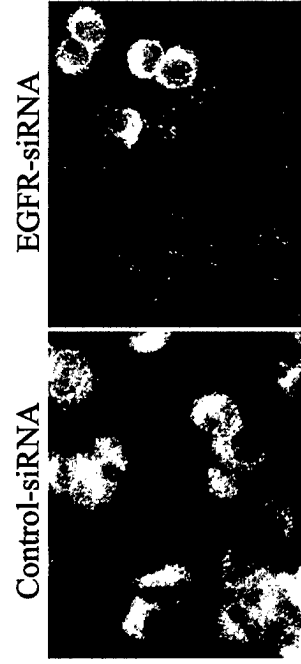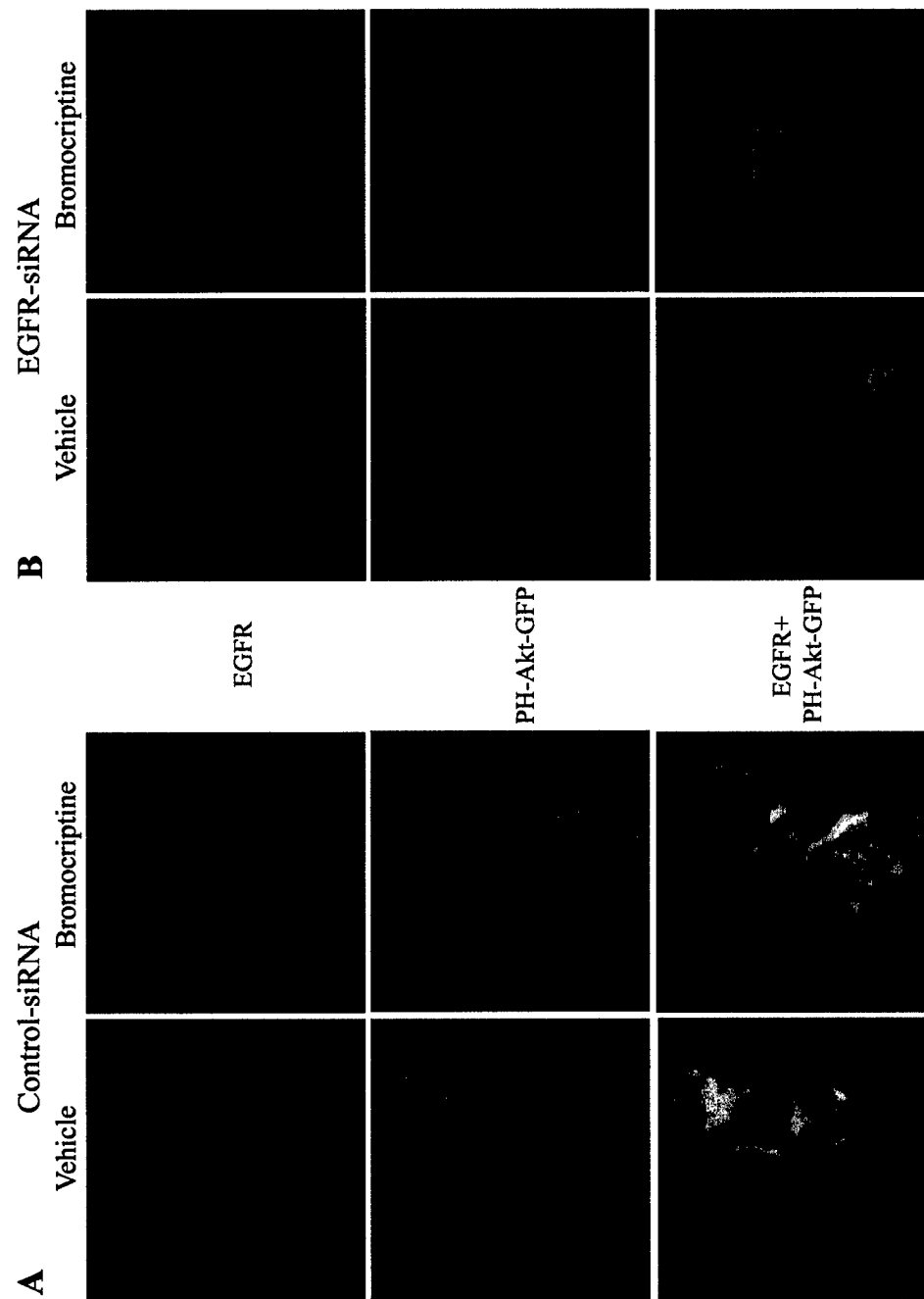
Figure 9

Figure 10

Figure 11